1989

Isolation, Characterization, and Symbiotic Effectiveness in Soybeans of Exopolysaccharide Mutants of Rhizobium Fredii USDA191.

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Order Number 9002155

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The Louisiana State University and Agricultural and Mechanical Col., 1989

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A Dissertation

submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in
The Department of Microbiology

by
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May, 1989

ACKNOWLEDGEMENT

I am grateful to my parents, wife, and daughter. They had hard times during my work on this dissertation.

My sincere thanks go to Dr. R. C. Gayda, my research advisor. Throughout this research he showed patience and generosity. He shared his precious time with me in giving advice and in discussing the results. If it had not been for his support, this research could not have been completed.

My special thanks go to Dr. H. D. Braymer and Dr. E. C. Achberger. Under complicated situations, they helped and encouraged me to come back to LSU and to continue my career. I became familiar with bacteriophage M13 and lambda because Dr. Braymer gave me theoretical and technical advice. His gift of lambda NM1149 phage is greatly appreciated. Dr. Achberger's encouragement for the last 5 years supplied me with immeasurable amounts of energy which enabled me to overcome some difficulties.

I also express my thanks to my advisory committee. Drs. D. S. Shih, M. D. Socolofsky, and J. W. Hoy for their valuable comments, corrections, and questions. The Electron Microscopy course taught by Dr. Socolofsky was enjoyable and made a great contribution to this research.

I am also indebted to Drs. M. E. Orlowski, M. C. Henk, and R. Tully. Dr. Orlowski was always willing to talk and listen to me and he was a positive supporter during my stay at LSU. Cindy Henk always did more work for me than I expected. She will all the time remind me of what a nice person can be. Especially, I would like to thank her for photography work. Dr. Tully donated to us soybean seeds and *Rhizobium* strains without which this research could not have been started.

I would also like to send thanks to L. A. Simmons for her advice and comments about lambda phage. They were very helpful for my research. In addition, thanks are extended to Yong-Hwan Lee for his statistical analysis of the nodulation experiments.

Finally, I would like to share my pleasure with my friends, Li-Dao Ke, Soo-Ho Kim, Sung-Ho Shin, and Yang-Bong Lee. Li-Dao Ke, a gentleman from China, did not care about age differences. Through my friendship with him I was encouraged. Soo-Ho Kim, Sung-Ho Shin, and Yang-Bong Lee spent much of their valuable time helping me. Whenever I needed their help, they did their best.

Y. H. Ko

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ABSTRACT

Chapter 1

Production of exopolysaccharides by *Rhizobium* has been linked with efficient invasion and nodulation of leguminous plant roots by the bacteria. Exopolysaccharide-deficient (*exo*) mutants of *Rhizobium fredii* USDA191 were isolated by Tn5-insertion mutagenesis. Five phenotypically unique *exo* mutants were investigated for exopolysaccharide synthesis and their ability to nodulate soybeans. The exopolysaccharides produced by these mutants were analyzed for polysaccharide composition by column chromatography and thin layer chromatography. Two mutants designated *exo3* and *exo5* were deficient in both the neutral glucan and exopolysaccharide synthesis, but still made effective nodules on *Glycine max* cv. Peking, although with decreased efficiency. The remaining three mutants (*exo*1, *exo*2, and *exo*4) synthesized neutral glucans at various levels compared with wild type and exhibited partial exopolysaccharide-deficiencies. One of these *exo* mutants, *exo*4, induced nodules on soybean, Peking. These data imply that neither exopolysaccharides nor neutral glucans are necessary for the formation of spherical nodules by *R. fredii.*

Chapter 2

Rhizobium fredii USDA191 is a fast-growing symbiont that nodulates soybeans as well as slow-growing *Bradyrhizobium* species. *R. meliloti exo* DNA clones (obtained from Graham Walker) were introduced by triparental plasmid matings into five *exo* mutants of *R. fredii* USDA191. These *exo* mutants of *R. fredii* each exhibited a unique Tn5 insertion pattern and a depressed exopolysaccharide synthesis. In two *R. fredii exo* mutants, exopolysaccharide expression was restored by introduction of *R. meliloti exo* DNA. *R. fredii* YKL288 (*exo*4) was complemented for exopolysaccharide synthesis when plasmid pD56 (*exo*F/B) but not plasmid pD2

(*exo*B) was introduced into it, and *R. fredii* YKL293 (*exo*5) was complemented for exopolysaccharide production when it contained plasmid pD15 (*exo*C). Significantly, plasmid pLYK5293 containing DNA sequences flanking the Tn5 of YKL293 hybridized with DNAs of plasmid clones pD56 (*exo*F/B), pD2 (*exo*B), and pD5 (*exo*D) of *R. meliloti*. Putative wild type *exo*5 genes also were cloned into a phage lambda NM1149 using plasmid pLYK5293 as a probe DNA. The data suggest the existence of common pathways for exopolysaccharide synthesis in *R. fredii* as well as in *R. meliloti*. The data also suggest a possible linkage in *R. fredii* USDA191 of *exo*C and *exo*D gene homologues of *R. meliloti*.

INTRODUCTION

Rhizobium species are classified based upon their ability to nodulate the roots of specific leguminous plants. Recently, they have been reclassified into four fast-growing species and one slow-growing species (7). All rhizobia, with few exceptions, have very narrow plant host ranges (67). In some cases, a bacterial symbiont is specific for a particular plant cultivar (35). This stringent host specificity of rhizobia is believed to be controlled at two levels: the initiation of nodule organogenesis and the invasion of plant by the bacteria.

Recognition between the plant and its corresponding bacterial symbiont occurs through cell surface and diffusible metabolite interactions (20,34,47,). Binding of rhizobia to the plant root hairs is believed to be by chance collision (54) and by chemotaxis (30). Following bacterial binding to the root hairs, there are physical contacts between the bacteria and the plant cell surfaces that are both specific and nonspecific in nature (17). Plant lectins and capsular polysaccharides (CPS) have been suggested to be the bridging polymers for this attachment (8,28,42,51,52,62). However, this involvement of lectins and CPS in binding is not applicable to all *Rhizobium* symbiosis.

The bacterial binding induces the plant root hairs to become deformed and the bacteria become entrapped within these root hairs. This process is called shepherd's crook formation (23). Plant cell penetration by the bacteria requires infection thread formation, even though rare intercellular penetration is observed (26,55,57). Infection threads are tubular pathways through which the bacteria can travel without evoking plant defense responses. The formation of an infection thread requires degradation of the preexisting plant cell wall and deposition of new material at the site of infection (12). The formation of infection threads and nodule meristem development are independent events. Initiation of nodule development

can occur without the presence of bacteria within the plant roots, through the actions of diffusible plant growth homones (9,16,26,44,45); however, without the bacteria, nodule development stops soon after formation of a small pseudonodule.

There are two types of root nodules which are formed by *Rhizobium* species and in which atmospheric nitrogen is fixed into ammonia. They differ not only in shape but also in the process of nodule development (33,56). Temperate legumes, such as clover, alfalfa, and, pea make indeterminate, cylindrical type nodules. Tropical legumes such as soybean, bean, and cowpea make determinate, spherical type nodules. The process for mature nodule formation takes different strategies between the determinate and indeterminate nodule types (33,56). For indeterminate nodule development, plant cell division is localized within the meristem, and these newly divided cells are infected by extensive branching of infection threads. For determinate nodule development, plant cell division occurs within the nodule through divisions of preinfected cells. In addition to the differences in nodule shapes, the bacteroids (the differentiated bacteria) within the indeterminate nodules exist in irregular polymorphic cell shapes, whereas those bacteroids within determinate nodules have a uniform shape of rods or swollen rods. The bacteria within the nodule remain separated from the plant cytoplasm by a peribacteriod membrane.

The genes of *Rhizobium* involved in nodulation can be divided into common (*nod*) and host specific (*hsn*) genes. The common *nod* genes (*nod*ABC) are interchangeable between *Rhizobium* species (20,27,41), but the regulation of this operon is dependent on the interaction with a plant host specific factor (11). Each plant species secretes its own specific flavonoid that activates *nod* operon expression in conjunction with the rhizobia *nod*D gene product (4,22,36,53). Other bacterial genes involved in nodulation are also classified as host specific, because they do not share any DNA homology with other rhizobia and can not complement

each other. Some of them include genes involved in exopolysaccharide synthesis and modification (4,37).

Rhizobium species produce extraordinarily large amounts of exopolysaccharides which are divided into capsular and extracellular polysaccharides. Extracellular polysaccharides are released to the culture supernatant, whereas capsular polysaccharides are tightly bound to the cells. They differ from each other only in the degree of polymerization (62). Exopolysaccharide production is dependent upon environmental growth conditions, such as carbon source, partial oxygen pressure, nitrogen compound availability and other culture medium composition (19,60,63,64). Moreover, genes involved in the regulation of exopolysaccharide synthesis, *psi* in *R. phaseoli* (10) and *exo*R/S in *R. meliloti* (19), have been identified.

R. meliloti 1021 is the most extensively studied species in terms of exopolysaccharide involvement in nodulation (24,25,26,44,45). Both *R. meliloti* 1021 and *R. fredii* USDA191 produce acidic exopolysaccharides though their composition is quite different. The structure and composition of *R. meliloti* exopolysaccharides has been shown to consist of an octasaccharide repeating unit (3). The repeating backbone contains seven β-linked glucoses and one β-linked galactose, and it is modified by pyruvylation, acetylation, and succinylation (32). The exopolysaccharides of *R. fredii* USDA191 have uronic acid, mannose, and in addition, galactose and glucose in about a 1:1:1:1 ratio. *R. fredii* exopolysaccharides are also pyruvylated (46). Exopolysaccharides from other fast and slow-growing soybean symbionts have uronic acids in common with USDA191 but are usually acetylated instead of being pyruvylated. The slow-growing rhizobia, such as *Bradyrhizobium japonicum*, also have their galactose polysaccharide residues methylated (38,51,52).

Calcofluor has been widely used as a stain for exopolysaccharides. It does not bind neutral glucans and low molecular weight acidic exopolysaccharides. Hence, exopolysaccharide-deficient (exo) mutants based upon calcofluor binding ability need to be interpreted with caution. Recently, calcofluor non-staining exopolysaccharide mutants were isolated from *R. meliloti* (32). Exopolysaccharides from the mutant had a repeating backbone of alternating glucose-galactose disaccharides which were acetylated and succinylated.

Exopolysaccharides have been hypothesized to be a determinant of host specificity (8), an inducer for correct plant responces (66), and a protectant of the bacteria from environmental hazards (58) and plant defense mechanisms (66). All of these exopolysaccharide functions are probably necessary for effective nodule development. The role of exopolysaccharides in the nodulation process is a significant area of investigation (1,2,18,26,43,59,65). Fast-growing *Rhizobium* species such as *R. meliloti* (26), *R. leguminosarum* (9), and *R. trifolii* (29) have been demonstrated to have common exopolysaccharide requirement for nodulation. Infection of legumes with *exo* mutants of the *Rhizobium* results in abnormal, pseudonodules which are devoid of bacteroids, hence ineffective in nitrogen fixation. On the other hand, *exo* mutants of *R. phaseoli* did not prevent the nodulation of beans even though a homologous *exo* mutation in *R. leguminosarum* did prevent the nodulation of peas (9). Moreover, *Rhizobium* NGR234, a broad host range strain, showed a different exopolysaccharide requirement for nodulation depending upon the host inoculated (6). *Exo* mutants of *R.* NGR234 made normal, effective nodules on determinate nodule forming hosts, whereas the same mutants induced small callus like structures on indeterminate nodule forming hosts. In addition, the nodulation defects of the *exo* mutants were alleviated by the addition of homologous exopolysaccharides in *R.* NGR234 and *R. trifolii* (18), but not in *R.*

meliloti (43). Therefore, the functional roles of exopolysaccharides in nodulation may be different with each specific symbiotic system.

The plant hosts also regulate the nodulation ability of the invading bacteria. The infection site on the root is restricted to only a specific area at the time of inoculation (6,13). Most nodules are found between the shortest emerging root hair area and root tips at the time of inoculation. In addition, bacterial inoculation slightly after nodule initiation do not affect the overall nodule number formed per plant.

Exo mutants of *R. meliloti* 1021 were isolated by Tn5 insertion mutagenesis and classified into several linkage groups (43,45,48). Five of them have been well characterized (43,45). *Exo*A, B, D, and F mutants were missing succinoglycans, but retained β-glucans. *Exo*C mutants were defective in both succinoglycan and β-glucan synthesis. All of the *exo* mutants except *exo*D showed a lack of nodule invasion and induced only pseudonodules. *Exo*D mutants synthesized a small molecular weight succinoglycan which was not normally found in the wild-type, but it could replace the large molecular weight exopolysaccharide in its symbiotic functions (32). Therefore, effective nodule formation was closely related with exopolysaccharide production in *R. meliloti*. The *ndv*A and *ndv*B mutants which did not produce glucans, were not able to make any infection threads (21). Interestingly, *Agrobacterium tumefaciens* also apparently could not attach to plant cells without neutral glucan production (14,15). Neutral glucans, which are normally within the periplasmic space of the rhizobia and also found in the culture media (69), have been proposed to be a bacterial signal that induces the formation of infection threads (1,31); however, others have suggested that they may also function as an osmotic stabilizer (50) or as a matrix for the synthesis of infection threads.

The effect of exopolysaccharides on nodulation of soybeans has not been reported. Soybean and cowpea groups occupy the largest area of cultivation among

leguminous plants. There are two genera of rhizobia which nodulate soybeans; *Rhizobium fredii*, a fast-grower, and *Bradyrhizobium japonicum*, a slow-grower. The slow growth rate (39) and polymorphism of the *Bradyrhizobium* species (61) have hindered studies of exopolysaccharide effects on its nodulation. *R. fredii* USDA191, a fast grower, grows on high salt media like other fast-growing species (68). It was isolated from a genetically unimproved soybean cultivar, Peking, in China and it can make nodules on commercial soybean cultivars (40).

In this study, exopolysaccharide-deficient mutants of *R. fredii* USDA191 were isolated by transposon Tn5 insertion mutagenesis. The relationship between exopolysaccharide production of these mutants and their nodulation ability on soybeans was investigated. In addition, the *exo* mutants of *R. fredii* were compared with *exo* mutants isolated by others from *R. meliloti* (43).

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CHAPTER 1.

Isolation and characterization of exopolysaccharide mutants of *Rhizobium fredii* USDA191: exopolysaccharide production and nodulation of soybeans.

Abstract

Production of exopolysaccharides by *Rhizobium* has been linked with efficient invasion and nodulation of leguminous plant roots by the bacteria. Exopolysaccharide-deficient (*exo*) mutants of *Rhizobium fredii* USDA191 were isolated by Tn5-insertion mutagenesis. Five phenotypically unique *exo* mutants were investigated for exopolysaccharide synthesis and their ability to nodulate soybeans. The exopolysaccharides produced by these mutants were analyzed for polysaccharide composition by column chromatography and thin layer chromatography. Two mutants designated *exo*3 and *exo*5 were deficient in both the neutral glucan and exopolysaccharide synthesis, but still made effective nodules on *Glycine max* cv. Peking, although with decreased efficiency. The remaining three mutants (*exo*1, *exo*2, and *exo*4) synthesized neutral glucans at various levels compared with wild type and exhibited partial exopolysaccharide-deficiencies. One of these *exo* mutants, *exo*4, induced nodules on soybean, Peking. These data imply that neither exopolysaccharides nor neutral glucans are necessary for the formation of spherical nodules by *R. fredii*.

Introduction

The induction of nitrogen fixing nodules on the roots of host legumes by *Rhizobium* and *Bradyrhizobium* bacteria is a multistep process involving interactions between both the host plant and its symbiont (see reviews 15,24,32,41). Bacterial lipopolysaccharides (10,11,47) and exopolysaccharides (14,21,22,36,37,61) have been implicated as early bacterial signals in nodule development. Exopolysaccharides are composed of capsular polysaccharides (CPS) and extracellular polysaccharides (EPS) that primarily differ in their degrees of polymerization (59). It has been demonstrated that exopolysaccharide-deficient (*exo*) mutants of several *Rhizobium* species do not form normal nitrogen-fixing nodules. Instead, these *exo* mutants often induced abnormal pseudonodules in the legume roots which were devoid of bacteroids (14,21,36,47,61). Two hypotheses for the functional role(s) of polysaccharides in bacterial invasion have been proposed: that they are necessary for the initiation of infection thread formation which allows the rhizobia to enter the plant (15,24) and/or that they allow growth of the bacteria within the infection thread without evoking the plant defense mechanisms (15,62). Thus, it is not clear whether exopolysaccharides are the direct inducer and/or the indirect protector in bacterial penetration process (52). Furthermore they may also act as a host specificity factor which binds plant lectin (see review 24,32).

The exopolysaccharides synthesized by various *Rhizobium* species can be largely divided into acidic polysaccharide and neutral polysaccharide components. Acidic exopolysaccharides are composed of hexoses and uronic acids (28,39,43,44,51). They are often pyruvylated in *R. leguminosarum* (51), *R. fredii* (39), and *R. trifolii* (22). In *B. japonicum*, the acidic

exopolysaccharides are methylated (28,43,44), while they are succinylated in *R. meliloti* (36). *R. meliloti* mutants, that lack the ability to succinylate their exopolysaccharides, were found only to induce pseudonodules devoid of bacteroids. Similarly, the acetylation of the exopolysaccharides of *R. trifolii* was found to be necessary for the attachment to clover roots. The neutral polysaccharide components contain the β-glucans (64). Approximately 5 to 10% of the total polysaccharides secreted by fast-growing *Rhizobium* species, such as *R. leguminosarum*, *R. phaseoli*, and *R. trifolii* are composed of β-2 linked glucans (64). The β-glucans are cyclic compounds with about 20 glucose residues. These molecules are indistinguishable from the β-glucans found to be synthesized by the plant tumor-inducing bacterium *Agrobacterium tumefaciens*. In fact, exopolysaccharide mutants of *A. tumefaciens* have been complemented by *R. meliloti* cloned *exo* genes and vice versa (9,17). There is a strong correlation relating β-1,2 glucan synthesis and plant attachment in both *Rhizobium* infection and *Agrobacterium* virulence (9).

Fast-growing rhizobia contain large molecular weight plasmids in which the common *nod* and *nif* genes have been linked (3,5,8,25,29). The common *nod*ABCD genes in *R. meliloti* have been identified (18) and shown to be regulated by a plant factor (45). In addition, host specific nodulation (*hsn*) genes have been identified that are required for early events such as host specific root hair curling and nodule initiation (*hsn*D) and infection thread growth (*hsn*ABC,27). It is not known whether these genes are involved in changing the conditions of the outer bacterial surface and affecting polysaccharide composition and/or synthesis.

Recently *nod*MN genes of *R. trifolii* have been identified as being involved in the acetylation of exopolysaccharide. The *nod*D2 gene of *R. japonicum*

USDA191 has been reported to regulate exopolysaccharide synthesis (4). In *R. meliloti*, six genetic loci involved in exopolysaccharide synthesis have been identified by gene cloning and complementation assays (20,37). Four of the *exo* mutations were mapped to megaplasmids within *R. meliloti*, and two mutations were located on the chromosome (21,29). Some of these *exo* mutants have been characterized (35).

In this paper, the role of *R. fredii* USDA191 exopolysaccharide synthesis in the nodulation of soybeans was investigated. *R. fredii* has a heterologous exopolysaccharide composition (39), which is quite different from that of the slow-growing *B. japonicum* or other fast-growing species like *R. meliloti.* To determine whether this heterogeneity of the polysaccharide was a prerequisite for *R. fredii* infectivity of soybeans, we isolated *exo* mutants and compared their ability to induce nodules with wild type.

Materials and Methods

Bacterial strains and plasmids: Strains and plasmids are listed in Table 1. *Escherichia coli* strains were maintained on Luria-Bertani (LB) medium (40) and *R. fredii* on mannitol-salt-yeast extract (MSY) medium (38). Antibiotics were added to the media at the following concentrations: 25 μg/ml for kanamycin sulfate and chloramphenicol for *E. coli* and 40 μg/ml for nalidixic acid, 50 μg/ml for kanamycin sulfate, 25 μg/ml for streptomycin sulfate, and 10 μg/ml for tetracycline hydrochloride for *R. fredii.*

Plants: Seeds of wild-type soybean, *G. soja* and *G. max* cv. Peking, were obtained from R. Tully (USDA-ARS, Beltsville, Maryland) and used for the nodulation tests.

Mutagenesis and screening *exo* mutants: Filter mating was done with a slight modification of the procedure of Hom et al. (26). Exponential cells of MM294A/pRK602 were mixed with cells of *R. fredii* YKL999, filtered through a nitrocellulose membrane, washed three times with 0.9% NaCl, and then the filters were incubated overnight on yeast extract-salt (YS) (38) agar plates at 28°C. Cells were washed off the filter with YS broth and dilutions were spread on MSY agar plate (38) containing nalidixic acid, kanamycin sulfate, and streptomycin sulfate (55). Small non-mucoid transconjugants were selected and purified by restreaking on same selection plates.

EPS production was quantified for each strain from late exponential cultures grown in mannitol-glutamate media (MG) (56) at 28°C. EPS was precipitated by the addition of two volumes of acetone to the culture supernatant (60). This precipitate was washed three times with a mixture of acetone and water (2:1), dried, and then dissolved in 1% H_2SO_4. Hexose content was determined by the anthrone

Table 1. Bacterial strains and plasmids

Strains and plasmids	Characteristics	References
Strains		
Escherichia coli		
SM10	C_{600}, *rec*A, Mu_c^+ with integrated plasmid RP4-2-Tc::Mu, Kn^r host of pSUP2021 and pSUP202	57
MM294A	*pro*-82 *thi*-1 *endo*A1 *hsd*R17 *sup*E44, host of pRK602	37
Rhizobium fredii		
USDA191	wild type	5
YKL224	Tn5 induced *exo*1 mutant of YKL999, Kn^r, St^r	this study
YKL257	„ *exo*2 „	„
YKL285	„ *exo*3 „	„
YKL288	„ *exo*4 „	„
YKL293	„ *exo*5 „	„
YKL999	Na^r derivative of USDA191	„
Plasmids		
pRK602	pRK2013 Nm::Tn9 containing Tn5, Kn^r, Cm^r	37
pSUP202	Ap^r, Cm^r, Tc^r	57
pSUP2021	pSUP202 Tc::Tn5, Ap^r, Cm^r, Kn^r	57

Abbreviations; Kn, kanamycin; St, streptomycin; Na, nalidixic acid; Nm, neomycin; Cm, chloramphenicol; Ap, ampicillin; Tc, tetracycline

method (42). The addition of two volumes of acetone does not precipitate neutral glucans (16).

Plant nodulation test: Method I. Seeds of *G. soja* were surface sterilized by soaking in concentrated H_2SO_4 for 15 min, rinsed five times with sterile H_2O for 5 min, washed with 50% ethanol for 5 min, then rinsed again three times with sterile water for 2 min each. Sterile seeds were germinated on 0.5% water agar at room temperature in darkness for 3-4 days. Seedlings were inoculated by dipping the roots in a late exponential phase culture of bacteria for 30 to 60 seconds, and then transplanted to vermiculite containing nitrogen-free salt medium (54). Plants were harvested after 4 weeks of incubation in a growth chamber under fluorescent light with a 14 hr photoperiod at 22°C (night) and 30°C (day). Every observable change in plant root tissue was counted. Four to five seedlings were inoculated with each strain and the experiment was performed twice.

Method II. Seeds of *G. max* cv. Peking were surface-sterilized by successive soaking in 50% ethanol (5 min) and 50% bleach (5min) and rinsing with copious amounts of sterile deionized water (49). Late exponential phase cultures of bacteria to be used as an inoculum were centrifuged, washed with water, and finally resuspended in water at a final concentration of 10^8 cells/ml. Surface-sterilized seeds were soaked in this inoculum for 1 hr, and planted in vermiculite containing nitrogen-free salt medium (54) with 0.5 ml of the inoculum. Subsequently, the seeds were incubated under darkness for 4-5 days and further grown under fluorescent light with a 14 hr photoperiod at 22°C (night) and 30°C (day). Plants were harvested after 4 weeks growth and examined for nodule formation. Four to five seedlings were inoculated with each strain and the experiment was performed twice.

Recovery of bacteroids from nodules: Nodules were excised from the roots and surface-sterilized in 50% Clorox for 2 min followed by five rinses with sterile water for 5 min. each. The nodules were then crushed in MSY medium (38) and plated on selective tryptone-yeast extract (TY) agar plates (31) to verify that the bacteroids recovered exhibited the same *exo* phenotype and antibiotic resistance as the original inoculum.

Scanning electron microscopy: Nodules were fixed with glutaraldehyde, ethanol-dehydrated, critical point dried, and then sputter-coated (48) and examined with a Hitachi S-500 Scanning Electron Microscope (SEM).

Preparation of EPS: Rhizobia were grown in MG broth (56) until late exponential phase at 30°C. Culture supernatants were obtained after centrifugation at 9,000 RPM (Sorvall SS34 or GS3 rotor) for 30 min. The supernatants were freeze-dried and dialyzed in 10 mM phosphate buffer (pH7.0) containing 0.02% sodium azide (elution buffer) at 4°C in dialysis tubing with a molecular weight cut-off (M.W.C.O.) of 1,000.

Preparation of CPS: Bacteria were grown in MG broth (56) until late exponential phase at 30°C with shaking. The cells were pelleted by centrifugation, washed once with phosphate-buffered saline [pH 7.2] (43), and then resuspended in the same buffer. CPS was extracted at 4°C for 6 days. The supernatants containing CPS after centrifugation were dialyzed (M.W.C.O.=1,000) against deionized water and concentrated by freeze-drying.

Separation and analysis of exopolysaccharide: EPS or CPS samples were separated by column chromatography. Bio-Gel A-5m (BioRad) columns (1.6 X 40 cm) were loaded with polysaccharide samples and eluted with elution buffer at 4°C. The elution rate was approximately 15 min/ml, and the fraction volume was 1 ml.

EPS samples were further fractionated by anion exchanger column when necessary. EPS samples were loaded on DEAE-Sephadex A-50 (Pharmacia) column (6.5 ml) at 25°C and washed with deionized water until no hexose was detected. Bound EPS was eluted from the column with 1M sodium acetate. For the identification of neutral glucans, the unbound fractions to the anion exchanger were hydrolyzed with 0.5N HCl at 100°C for 5 to 20 minutes (22). The acid hydrolysates were developed on silica gel thin-layer plate (Analtech) using a solvent mixture of isopropanol: acetic acid: water (27:4:9) and visualized with orcinol ferric chloride (Sigma) (6).

Plasmid separation and DNA hybridization: Plasmids were separated by agarose gel electrophoresis using an in-well lysis technique(30). DNA was transferred to nitrocellulose filters by the method of Southern (40) after partial depurination cleavage (61). Acid depurination before alkali denaturation reduced the blotting time from 48 hrs to 6 hrs, and increased the blotting efficiency (data not shown). Without acid depurination, complete transfer of DNA from agarose gel to the filter was impossible even after 48 hr of blotting. A biotin-labeled Tn5 probe (34) was prepared by nick-translating pSUP2021 DNA using a biotin-11-dUTP nucleotide (Bethesda Research Labs) (50). DNA-DNA hybridization and detection of hybrids were performed according to the manufacturer's procedure (BRL).

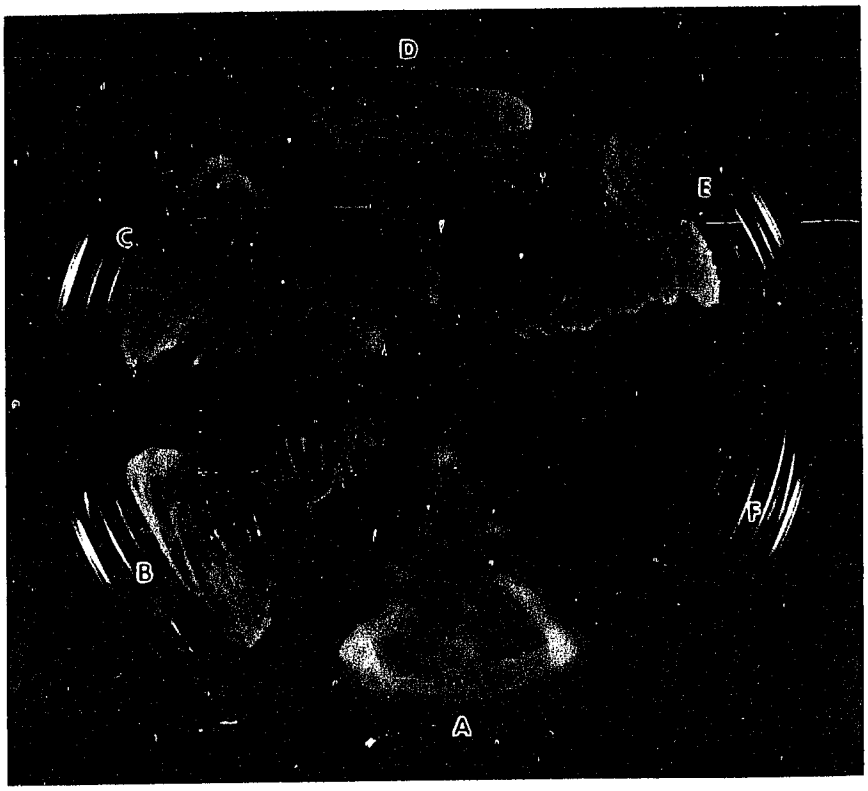
Results

Isolation of *exo* mutants.

Suicide plasmid pRK602 containing the transposon Tn5 was mobilized from MM294A/pRK602 to YKL999 by filter mating. From this mating, kanamycin resistant transconjugants (Tn5 insertion mutants) were obtained at a frequency of $1x10^{-5}$ per recipient. *Exo* mutants with a nonmucoid appearance on MSY agar plates were isolated at a frequency of about $1x10^{-3}$. Eighty-five *exo* mutants were isolated and, of these, five phenotypically unique, nonmucoid colonies were chosen for further study. The phenotypes of these five mutants on MSY plates are shown in Fig. 1. Wild type YKL999 colonies (Fig.1A) were highly mucoid in appearance and produced large, watery and opaque colonies. YKL224 (*exo*1) (Fig.1B) produced colonies that were non-mucoid and whiter in color than the wild-type strain. YKL224 produced only 4 % of the acetone precipitable EPS of wild-type. YKL257 (*exo*2) (Fig.1C) was slightly mucoid and produced 43% of the acetone precipitable EPS of the wild type. YKL285 (*exo*3) (Fig.1D) was more translucent and produced 12% of the amount of EPS compared to the wild-type strain. Strains YKL288 (*exo*4) (Fig.1E) and YKL293 *(exo*5) (Fig.1F) were nonmucoid and produced only 2% and 4%, respectively, of acetone precipitable EPS compared to the wild type. In addition, strain YKL293 grew slower and formed the smallest colonies on agar plates when compared with the other *exo* mutants. Both YKL288 and YKL293 colonies produced a greenish hue on MSY agar plates. Addition of galactose to MSY agar resulted in white colonies from strain YKL288 but did not affect the colony phenotype of strain YKL293.

R. fredii USDA191 contains at least three large plasmids of 65Kb, 229Kb, and >300Kb (3,24,52). We examined whether any of the five *exo* mutants contained Tn5 inserts within these plasmids. Bacteria were lysed by an in-well lysis technique

Fig. 1. Colony morphology of wild type and *exo* mutants of *R. fredii* USDA191 on MSY agar plate. A, YKL999(wt); B, YKL224(*exo*1); C, YKL257(*exo*2); D, YKL285(*exo*3); E, YKL288(*exo*4); F, YKL293(*exo*5)


D
E
C
F
B
A

(30). The three large plasmids were resolved by agarose gel electrophoresis and transferred to nitrocellulose filter. A biotin-labeled probe of pSUP2021 which contained Tn5 was hybridized to the DNA on nitrocellulose filters (Fig.2). Positive DNA hybridization signal was detected with the DNA from MM294A/pRK602 with the original conjugated plasmid (lane a) but was not detected with DNAs from any of the mutants or YKL999. Some positive hybridization signal could be detected on the top of the agarose gel (lanes c-g) where large chromosomal DNA fragments migrated. As a negative control hybridization with a pSUP202 probe was not detected (data not shown). Thus, we concluded that the Tn5 fragments were located on the chromosomes in all five *exo* mutants of *R. fredii.*

Nodulation ability of *the exo* mutants.

Nodulation ability of these five *exo* mutants was examined by infecting the roots of soybeans. The nodules that formed on the plant roots consisted of two types. The first class were typical spherical nodules that were large, determinate, and pink-pigmented. From these nodules, bacteroids were recovered after surface sterilization of the nodule. The second class of nodules were small, irregular in shape, and white with a vascular bundle in the center. No bacteroids could be recovered from these atypical nodules. Scanning electron micrographs of both nodule types are shown in Fig. 3. Under SEM, no bacteroids could be seen when the atypical "pseudonodules" were cross-sectioned (Fig.4A), whereas clusters of bacteroids were found inside the cortical layer of typical nodules (Fig.4B).

Table 2 presents the average number of nodules per plant. YKL293 (*exo5*) is the only mutant strain that did not make any typical nodules on *G. soja*. The other four mutant strains made typical nodules comparable to those induced by the wild-type strain YKL999. Bacteroids recovered from typical nodules were phenotypically the same in relation to EPS production and antibiotic resistance as the inoculating

Fig. 2. Plasmid separation on 0.6% agarose gel (A-G) and DNA-DNA hybridization with a Tn5 probe (a-g). A/a, MM294A/pRK602; B/b, YKL999(wt); C/c, YKL224(*exo*1); D/d, YKL257(*exo*2); E/e, YKL285(*exo*3); F/f, YKL288(*exo*4); G/g, YKL293(*exo*5).

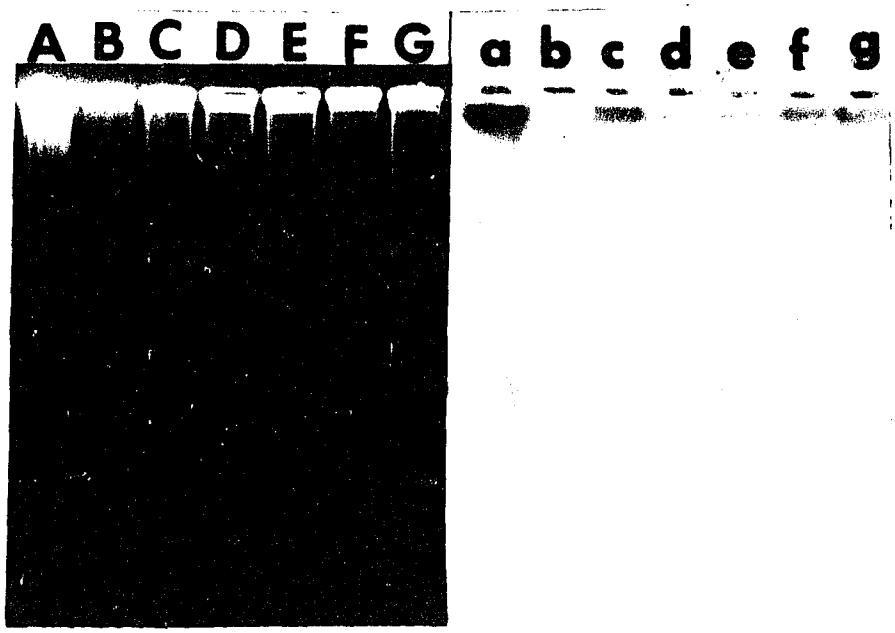

A B C D E F G
a b c d e f g

Fig. 3. Atypical and typical nodules made on *G. soja* by *R. fredii* USDA191 after 4 weeks growth. Pictures were taken by scanning electron microscope. A, atypical nodule; B, typical nodule.

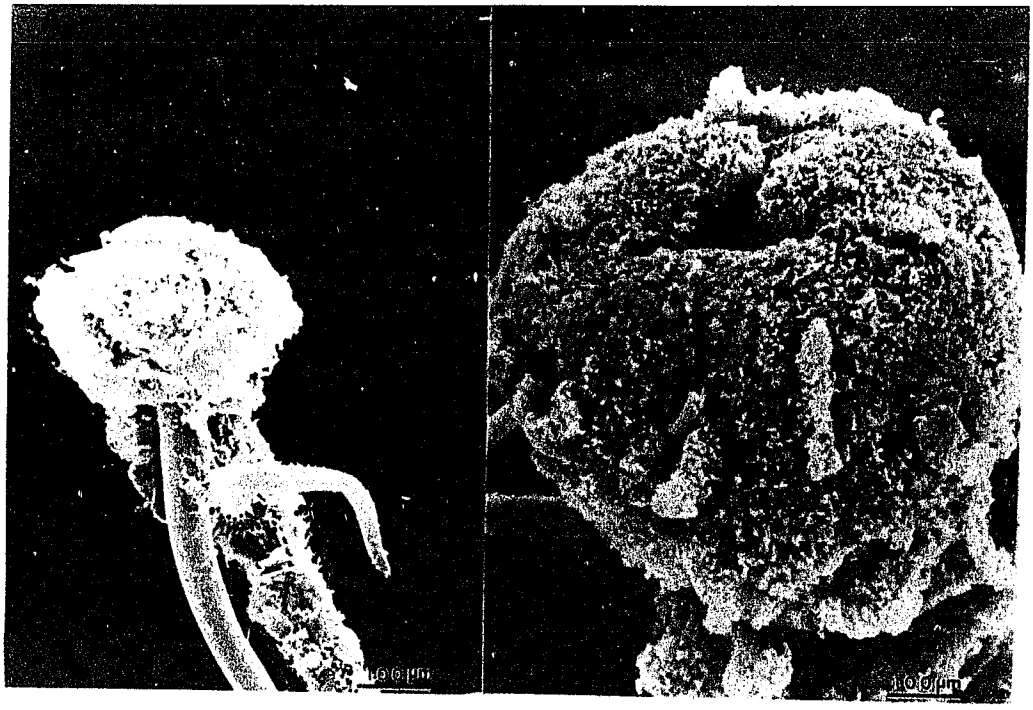
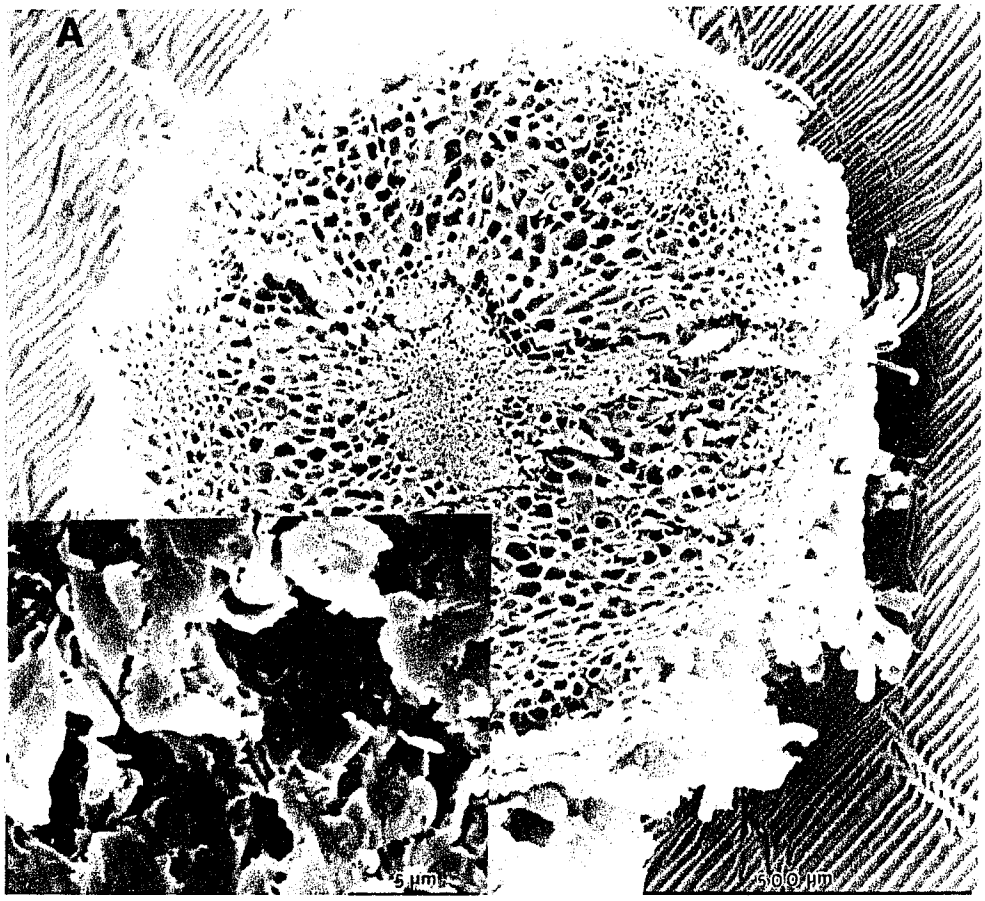

100 µm
100 µm

Fig. 4. Scanning electron micrographs of a cross-secton of nodules made on *G. soja* by *R. fredii* USDA191. A, atypical nodule; B, typical nodule; Inserts, enlargements of nodule centers.


A
5 µm
500 µm

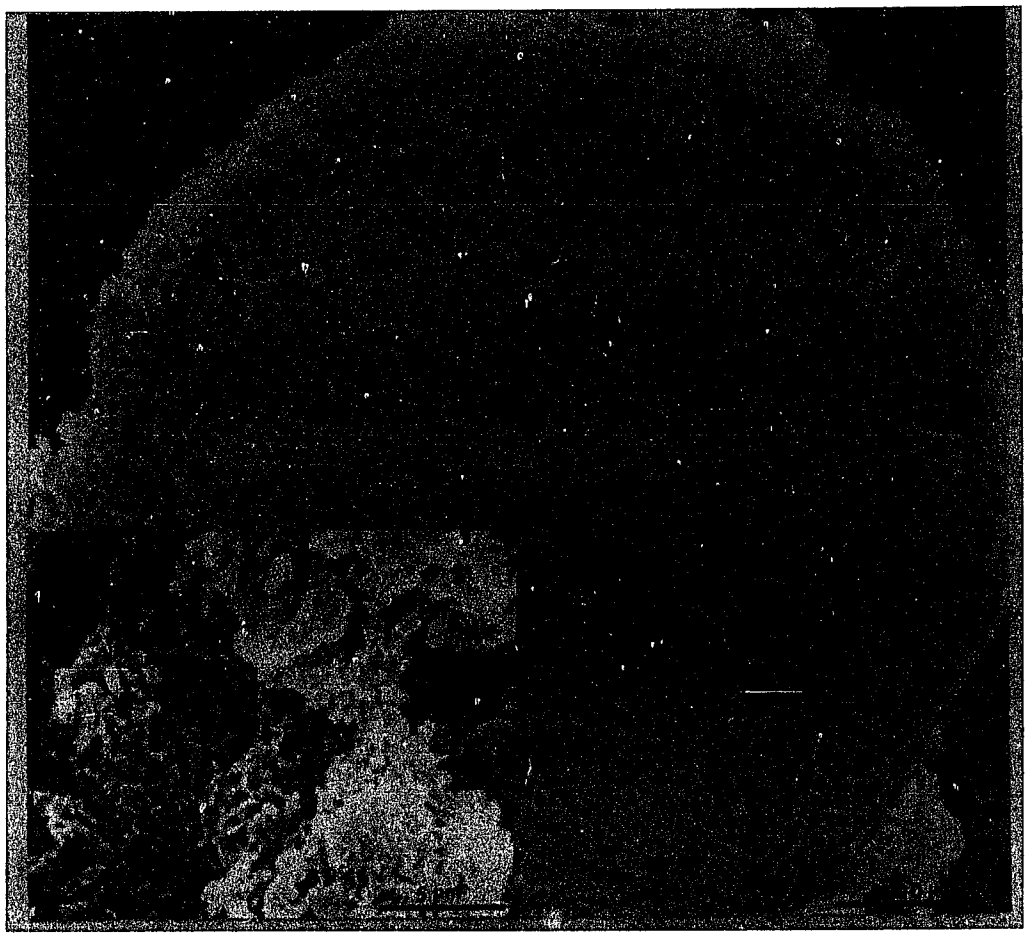


Table 2. Nodulation of soybeans and exopolysaccharide production by *R. fredii* USDA191 wild type and *exo* mutants.

strains	number of nodules per plant(T/A)* *G. soja*	*G. max*	neutral glucan fraction** (%/wt)	CPS*** (%/wt)	EPS**** (%/wt)
YKL999(wt)	1.0^{a} / 10	3.4^{c} / 0.0	100.0	100.0	100
YKL224(*exo*1)	1.2^{a} / 20	0.1^{f} / 2.1	235.0	N.D.	4
YKL257(*exo*2)	1.0^{a} / 32	0.1^{f} / 4.7	100.0	N.D.	43
YKL285(*exo*3)	1.3^{a} / 26	$1.3^{d,e}$/15.2	2.5	4.0	12
YKL288(*exo*4)	$0.4^{a,b}$/ 15	2.0^{d} / 3.4	25.0	N.D.	2
YKL293(*exo*5)	0.0^{b} / 16	$0.5^{e,f}$/ 9.4	1.0	4.2	4
control	0.0^{b} /0.0	0.0^{f} / 0.0	N.A.	N.A.	N.A.

Abbreviations; N.D., not determined; N.A., not applicable; wt, wild type

*Four to five seedlings of soybean were inoculated with each strain for two separate experiments and repeated once. The number indicates LS (least squares) means of typical (T) and average numbers of atypical (A) nodules per plant. LS means superscripted by the same letter are not significantly different (P=0.05) according to least significant difference tests performed on individual LS means.

** Neutral EPS fraction was estimated from the elution profile on Biogel A-5m column.

*** CPS was estimated from the elution profiles on Biogel A-5m column (Fig.8).

****EPS was determined by addition of two volumes of acetone to culture supernatant.

bacteria. The number of nodules per plant was typical for wild type *R. fredii* infection of soybeans (25). *Exo* mutant YKL293, as well as other strains including wild type, induced the formation of many atypical "pseudonodules" on the infected roots of *G. soja*

When the same strains were used as inocula on another soybean, Peking, all *exo* mutant strains tested made effective nodules but with less efficiency compared with the wild-type strain. The plants, inoculated with the wild-type strain, were relatively tall, healthy, and green after 4 weeks growth (Fig.5). The uninoculated control plants began to turn yellow as soon as the cotyledons fell off. In contrast to *G.soja,* Peking inoculated with YKL285 (exo3) and YKL293 had typical nodules. Furthermore, the number of nodules was less than the number formed by the wild-type bacteria, and, in the case of YKL293, bacterial infections did not result in the development of an effective nodule on every plant.

Exopolysaccharide analysis of the *exo* mutants.

To analyze the EPS synthesized by each mutant, EPS from the culture supernatants were separated by size on a Bio-Gel A-5m column (Fig.6). Supernatant from wild type YKL999 fractionated as a set of heterogenous polysaccharides; large M.W. material eluted in fractions just after the void volume up to an elution volume of 85 ml (small M.W. material), with a major peak (A_{620}) observed between elution volume 65 and 75 ml (Fig.6A). In contrast to the wild type, all the *exo* mutants possessed polysaccharide that was much less heterogenous. EPS extracted from strain YKL224 (*exo*1) (Fig.6B) eluted as one sharp peak between elution volumes 65 and 75 ml. YKL257 (*exo*2) (Fig.6F) and YKL288 (*exo*4) (Fig.6E) synthesized polysaccharides that were similar to YKL224 polysaccharide but with the addition of two minor peaks of large M.W. polysaccharide fractions. *Exo* mutants, YKL285 (*exo*3) (Fig.6D) and YKL293

Fig. 5. Nodulation of *G. max* cv. Peking by *R. fredii* USDA191. a, control without bacterial inoculation; b, USDA191; c, YKL999(wt); d, YKL285(*exo*3); e, YKL288(*exo*4); f, YKL293(*exo*5). Plants were grown for 4 weeks in nitrogen-free salt medium (A) and roots were cut off from each plant to examine for nodules (B). Note that the plant without a typical nodule (f) is like the uninoculated control (a).

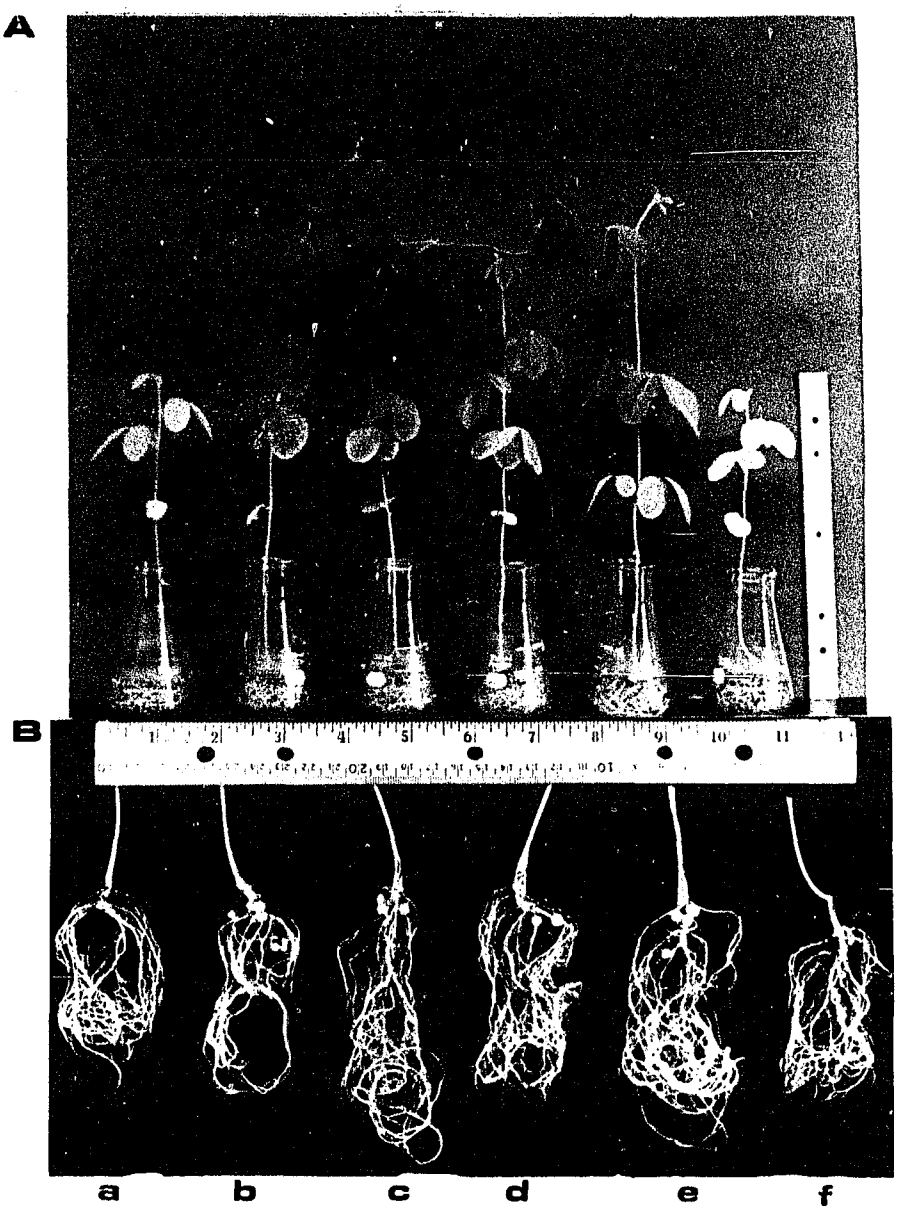
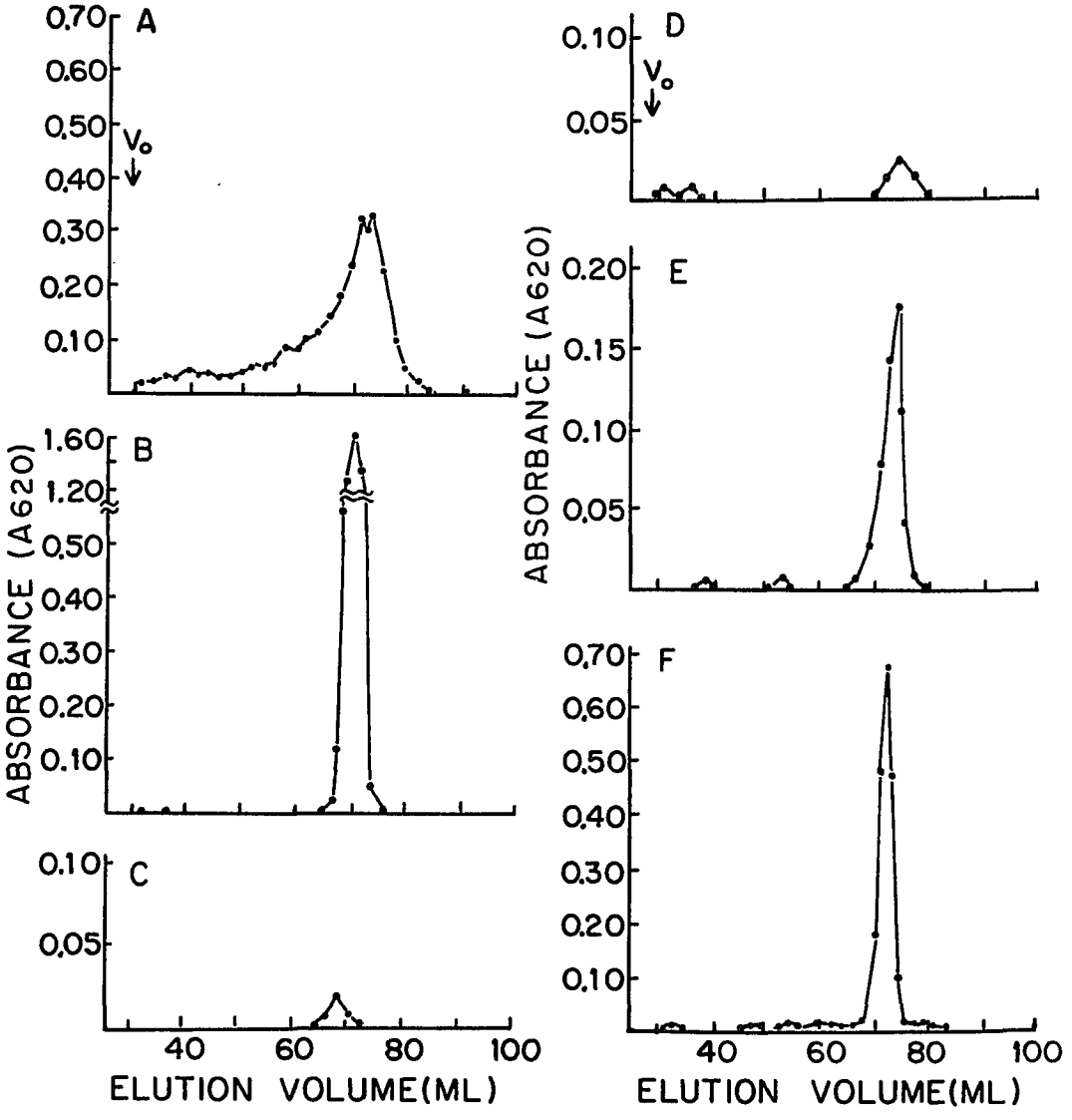

A
B
a
b
c
d
e
f

Fig. 6. Elution profiles of EPS separated on BioGel A-5m column (1.6 x 40 cm). Vo indicates void volume of the column. A, YKL999(wt); B, YKL224(*exo*1); C, YKL293(*exo*5); D, YKL285(*exo*3); E, YKL288(*exo*4); F, YKL257(*exo*2).


A
B
C
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ABSORBANCE (A620)
ELUTION VOLUME(ML)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
1.60
1.20
0.15
0.05
40
60
80
100

(*exo5*) (Fig.6C), had litte polysaccharide even within the major peak (the A_{620} was maximally < 0.05). This residual peak had a brownish color instead of the normal greenish-blue color observed in YKL999, YKL224, YKL257, YKL288, which suggests that it is not likely a hexose fraction. YKL285 did have a trace amount of large M.W. polysaccharide eluting after the void volume.

Since EPS consists of both acidic and neutral components, the polysaccharides were further fractionated on DEAE-Sephadex columns to examine synthesis of both types. When EPS of the wild type, YKL999, was analyzed, less than 5% of the total EPS was neutral polysaccharide. On the other hand, EPS of strain YKL224 (Fig.6B) when separated into two fractions on DEAE-Sephadex (Fig.7A) consisted of 55% neutral and 45% acidic polysaccharide. EPS of another *exo* mutant strain, YKL288, was composed primarily of neutral polysaccharide which was 70% of the total EPS (Fig.7B).

When the neutral EPS fraction was hydrolyzed in 0.5N HCl at 100°C, the complete acid hydrolysate showed only one spot corresponding to a glucose standard on silica gel thin-layer plates. The partial hydrolysate, on the other hand, was composed of glucose polymers with different degrees of polymerization. Thus, the neutral EPS fraction is most likely a glucan. In Table 2, the amount of neutral glucan produced by the *exo* mutants is presented for comparison with the wild type.

Since the polysaccharide differences between YKL285 and YKL293 strains were not evident from EPS analysis, the production of CPS was analysed. CPS from the wild-type strain (Fig.8A), when analyzed on a BioGel A-5m column, migrated as a single peak of low M.W. polysaccharide. When YKL285 (Fig.8B) and YKL293 (Fig.8C) were analyzed for CPS, both mutants exhibited very little CPS, if any. Thus, both mutants were also deficient in CPS.

Table 2 compares exopolysaccharide production with the ability of the *exo* mutants to nodulate soybeans. Of interest were the severe *exo* mutants YKL285

Fig. 7. Elution profiles of EPS separated on DEAE-sephadex A-50 column (6.5 ml). Arrow head indicates the point of addition of 1M sodium acetate. A, YKL224(*exo*1); B, YKL288(*exo*4)

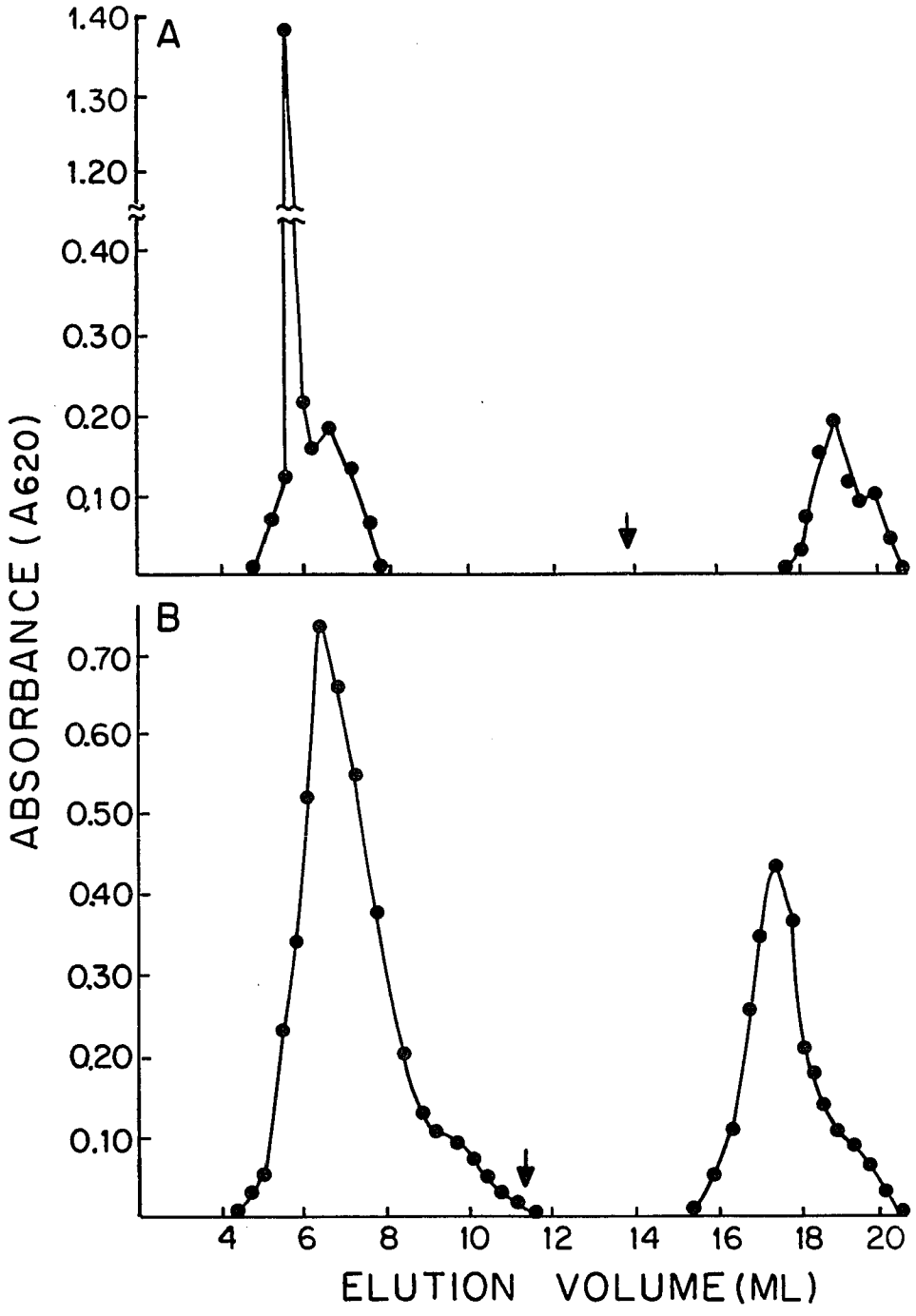
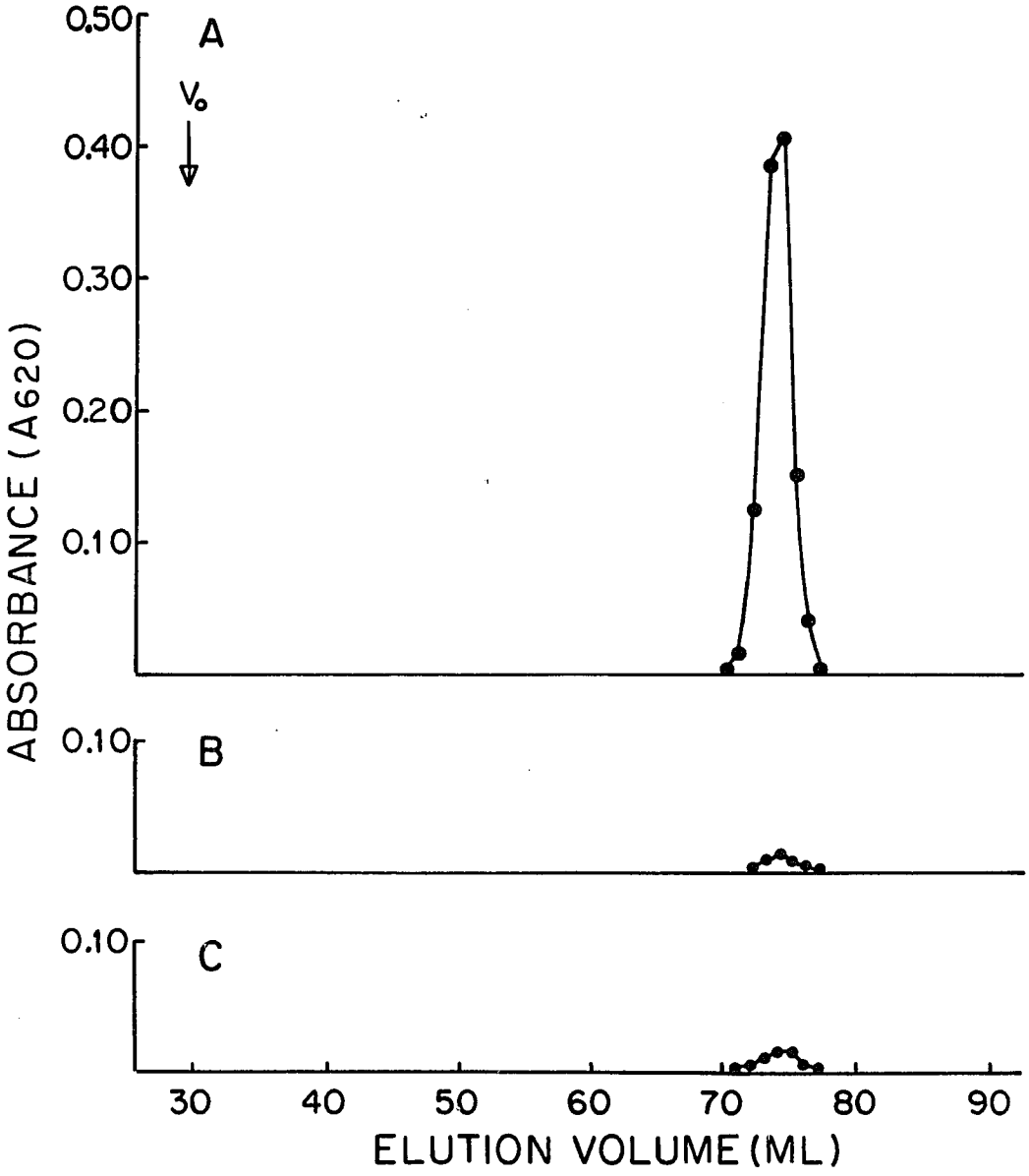

A
B
1.40
1.30
1.20
0.40
0.30
0.20
0.10
0.70
0.60
0.50
0.40
0.30
0.20
0.10
ABSORBANCE (A620)
4
6
8
10
12
14
16
18
20
ELUTION VOLUME (ML)

Fig. 8. Elution profiles of CPS separated on BioGel A-5m column. Vo, void volume of the column. A, YKL999(wt); B, YKL285(*exo3*); C, YKL293(*exo5*).


0.50
A
V_o
0.40
0.30
0.20
0.10
ABSORBANCE (A620)
0.10
B
0.10
C
30
40
50
60
70
80
90
ELUTION VOLUME (ML)

(*exo*3) and YKL293 (*exo*5) which were deficient in both EPS and CPS. YKL285 was able to form effective nodules on both *G. soja* and *G. max* Peking. YKL293 was able to form effective nodules on *G. max* but not on *G. soja*. *R. fredii* USDA191 was originally isolated from *G. max* Peking (33), and this plant cultivar appeared to be a preferred host. In addition, a mutant YKL288 (*exo*4) that produced only 25% of the normal neutral glucan and that synthsized little of the large M.W. polysaccharides showed a decreased ability to nodulate *G. max*. A surprise was the extremely poor nodulation abilities on *G. max* of YKL224 (*exo*1), a mutant with EPS enriched for neutral glucan, and YKL257 (*exo*2), a mutant that produced about half the normal amount of EPS.

Discussion

Exopolysaccharide-deficient (*exo*) mutants of *R. fredii* USDA191 were isolated by transposon Tn5 insertion mutagenesis. Four *exo* mutants of *R. fredii*, YKL224 (*exo*1), YKL257 (*exo*2), YKL285 (*exo*3) and YKL288 (*exo*4), made typical nodules on *G. soja*. Only one mutant, YKL293 (*exo*5), was found not to be able to nodulate this plant species. *Exo*5 mutation is probably pleiotropic because it makes the mutant cells grow slower than the other *exo* mutants and wild-type. Interestingly, it was observed that infection of *G. soja* with *R. fredii* induces the production of a large number of atypical nodules (Table 2), that is, plant differentiation without bacterial invasion. Even though *R. fredii* USDA191 is a wide host range symbiont (53), it is likely that *G. soja* is not a preferred plant host. Upon testing strains YKL293 and YKL285 for nodulation on a different soybean species, *G. max*, both *exo*5 and *exo*3 mutants were able to make effective nodules. Analysis of exopolysaccharides produced by YKL293 and YKL285 revealed that they were each deficient in EPS, CPS, and neutral glucan. Thus, this data suggest that exopolysaccharide synthesis is not by itself essential for *R. fredii* to induce an effective nodule on soybean roots. The exopolysaccharide deficiency does lower the average number of nodules produced per plant, although the average number of nodules on *G. max* induced by *exo*4 mutant (YKL288) appeared to be less affected by its exopolysaccharide deficiency (compare 2.0 vs 3.4 nodules/plant, Table 2). The nodulation ability of *exo*1 (YKL224) and *exo*2 (YKL257) mutants was very poor on *G. max*. It is likely that *exo*1 has a mutation in cell membrane structure or polysaccharide polymerization, because periplasmic neutral glucan is secreted in excess in this mutant. And *exo*2 may have a mutation in pyruvylation of exopolysaccharides, because its exopolysaccharide synthesis was slightly decreased compared with wild type.

Why exopolysaccharide deficiency in *R. fredii* does not affect the ability to nodulate the host as severly as *exo* mutants of *R. meliloti* is the question. In *R. meliloti*-alfalfa symbiosis, exopolysaccharide synthesis was essential for effective nodule formation. Furthermore without succinoglycan, no infection thread was made, or it was aborted at initiation stage (36). Similarly, *exo* mutants of *R. leguminosarum* were found not to make effective nodules on peas; however, the same *exo* mutation within *R. phaseoli* did not prevent nodulation of beans (7). More decisively, Chen, et al. (12) tested the same Tn5 induced mutants of *R.* NGR234, a broad host range strain, for their nodulation abilities on determinate and indeterminate nodule forming hosts. The same *exo* mutants made normal to small, abnormal nodules on determinate hosts, but induced small callus-like structures on indeterminate hosts. Thus, a major factor in the effect of exopolysaccharide on nodulation may be the plant genotypes.

R. fredii USDA191 makes determinate nodules on soybeans. Our data suggest that there is no absolute requirement of exopolysaccharide for effective nodulation. Rather, it appears that exopolysaccharide deficiency just reduced bacteria's successful penetration. We propose that exopolysaccharide requirement is not as essential for determinate nodule formation as for indeterminate nodule formation. First, determinate and indeterminate nodules, whose types are determined by plant host have different strategies for development (46). Secondly, the susceptibility of host appears to vary even within same plant species (13). In severe case, nodulation of *Phaseolus vulgaris* by *R. fredii* showed host genotype dependent efficacy (53). Alternatively, exopolysaccharide may only play an indirect role, that of allowing better binding to host specific lectin (24,32) or protecting the bacterial cells from environmental hazards (52). Thus, sufficient exopolysaccharide may increase cell survival and host-symbiont contacts (11).

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CHAPTER 2.

Complementation of Tn5 induced exopolysaccharide-deficient mutants of *Rhizobium fredii* USDA191 by *Rhizobium meliloti exo* genes

Abstract

Rhizobium fredii USDA191 is a fast-growing symbiont that nodulates soybeans as well as the slow-growing *Bradyrhizobium* species. *R. meliloti exo* DNA clones (obtained from Graham Walker) were introduced by triparental plasmid matings into five exopolysaccharide-deficient (*exo*) mutants of *R. fredii* USDA191. These *exo* mutants of *R. fredii* each exhibited a unique Tn5 insertion pattern and a depressed exopolysaccharide synthesis. In two *R. fredii exo* mutants, exopolysaccharide expression was restored by introduction of *R. meliloti exo* DNA. *R. fredii* YKL288 (*exo*4) was complemented for exopolysaccharide synthesis when plasmid pD56 (*exo*F/B) but not plasmid pD2 (*exo*B) was introduced into it, and *R. fredii* YKL293 (*exo*5) was complemented for exopolysaccharide production when it contained plasmid pD15 (*exo*C). Significantly, plasmid pLYK5293 containing DNA sequences flanking the Tn5 of YKL293 hybridized with DNAs of plasmid clones pD56 (*exo*F/B), pD2 (*exo*B), and pD5 (*exo*D) of *R. meliloti*. Putative wild type *exo*5 genes also were cloned into a phage lambda NM1149 using plasmid pLYK5293 as a probe DNA. The data suggest the existence of common pathways for exopolysaccharide synthesis in *R. fredii* as well as in *R. meliloti*. The data also suggest a possible linkage in *R. fredii* USDA191 of *exo*C and *exo*D gene homologues of *R. meliloti*.

Introduction

Fixation of atmospheric dinitrogen by legume-*Rhizobium* symbiosis is a significant area of research in agriculture. Formation of nitrogen-fixing nodules requires specific interactions between rhizobia and leguminous plants. The role of rhizobial exopolysaccharides in the plant-microbe interactions has been widely investigated (see reviews; 11,15,20). *Rhizobium fredii* USDA191 nodulates soybeans like *Bradyrhizobium* species even though the composition and structure of their exopolysaccharides are not identical (23,30). Most surprisingly, the exopolysaccharide composition and structure differ even within *Bradyrhizobium* species (17). *R. fredii* USDA191 also has similarities with other fast-growing rhizobia such as *R. meliloti.* They both produce neutral glucans in addition to acidic polysaccharides (39), grow in high salt medium (38), and contain large molecular weight plasmids on which symbiotic genes are located (2,4,7). In addition, *nif* and *nod* genes are conserved between the bacterial species (2,7). *R. fredii* USDA191 may be an evolutionary common ancestor for fast and slow-growing *Rhizobium* species (25).

The production of exopolysaccharides by *R. meliloti* has been characterized primarily by Graham Walker and co-workers (21,22,25). The structure and composition of the exopolysaccharides *of R. meliloti* are known (14). However, there is only one report describing the composition of the exopolysaccharides *of R. fredii* USDA191 (23). Their composition is quite different from that of *R. meliloti* exopolysaccharides, because they contain uronic acid and mannose, in addition to, glucose and galactose that constitute the backbone of *R. meliloti* exopolysaccharides. However, it is probable that common pathways for exopolysaccharide synthesis exist in *Rhizobium* species, because Gram-negative bacteria appeared to share lipid-bound oligosaccharide intermediates in capsular polysaccharide synthesis (35,36).

In this paper, five unique Tn5-insertion *exo* mutants of *R. fredii* USDA191, which have been previously described in Chapter 1, were tested for gene complementation by plasmids containing *exo* genes of *R. meliloti*. In addition a plasmid, containing the sequences flanking the Tn5 insertion of strain YKL293, was isolated and then used to test for cross DNA hybridization with the plasmids containing the *exo* genes of *R. meliloti*.

Materials and methods

Bacterial strains, plasmids, and phages: Strains, plasmids, and phages are listed in Table 1. *Escherichia coli* strains were grown and maintained in Luria-Bertani (LB) media (26) and *Rhizobium* strains in mannitol-salt-yeast extract (MSY) media (24) unless otherwise stated. Antibiotic concentrations used were 25 μg/ml for kanamycin sulfate, streptomycin sulfate, chloramphenicol,and carbenicillin, 10 μg/ml for tetracycline hydrochloride, and 40 μg/ml for nalidixic acid. A higher concentration (50 μg/ml) of kanamycin sulfate was used for *Rhizobium fredii.*

Enzymes and chemicals: All enzymes except those described below were purchased from Bethesda Research Laboratory (BRL). DNaseI, RNaseA, proteinase K, lysozymes, 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside (X-gal), and hexadecyltrimethylammonium bromide (CTAB) were from Sigma Chemical Co. Sodium dodecyl sulfate (SDS) and cesium chloride (CsCl) were obtained from Bio-Rad Laboratories. Low melting-temperature agarose (FMC BioProducts) and ethidium bromide (EtBr) (International Biotechnologies Inc) were used for DNA preparations.

Preparation of extracellular polysaccharide (EPS): Rhizobia were grown in 500 ml of mannitol-glutamate (MG) broth (33) until late exponential growth phase at 30°C. Culture supernatants were obtained by centrifugation at 9,000 RPM (Sorvall GS3 rotor) for 30 min. The supernatants were then freeze dried, redissolved in minimal volume of water, and dialyzed in 10mM phosphate buffer (pH7.0) containing 0.02% sodium azide (elution buffer) at 4°C in dialysis tubing with a molecular weight-cut off (M.W.C.O.) of 1,000.

Table 1. Bacterial strains, plasmids, and phages

Strains and plasmids	Characteristics	References
Strains		
Escherichia coli		
SM10	C_{600} *rec*A Mu_c^+ with integrated plasmid RP4-2-Tc::Mu,Kn^r, host of pSUP2021 and pSUP202	34
MM294A	*pro*-82 *thi*-1 *end*A1 *hsd*R17 *sup*E44, host of pRK602	22
HB101	*pro leu thi lac*Y *rps*L20(st^r) *endo*I *rec*A *hsd*S20(r^-,m^-), host of pRK2013 and pLAFRI clones	10
NM514	*hsd*R *lyc*7, host of lambda NM1149 clones	16
SM32	*lon gal*E *sul*A *str*A, host of lambda NM1149	16
DH5α	*hsd*R *rec*A1 Δ(*arg*F-*lac*ZYA) U169 ϕ80d*lac*ZΔM15, host of pUC19	18
Rhizobium fredii		
USDA191	wild type	4
YKL224	Tn5 induced *exo*1 mutant of YKL999, Kn^r, St^r	this study
YKL257	„ *exo*2 „	„
YKL285	„ *exo*3 „	„
YKL288	„ *exo*4 „	„
YKL293	„ *exo*5 „	„
YKL999	Na^r derivative of USDA191	„
Plasmids		
pRK602	pRK2013 Nm::Tn9 containing Tn5, Kn^r, Cm^r	22
pRK2013	helper, Kn^r	10
pSUP2021	pSUP202 Tc::Tn5, Ap^r, Cm^r, Kn^r	34
pSUP202	without Tn5, Ap^r, Cm^r, Tc^r	34
pD56	pLAFRI with *exo*F/B from *R. meliloti*, Tc^r	22
pD2	„ *exo*B „	„
pD5	„ *exo*D „	„
pD34	„ *exo*A „	„
pD15	„ *exo*C „	„
pUC19	Ap^r, cloning vector	37
pLYK5293	a derivative of pUC19 Ap^r, Kn^r	this study

(continued)

Phages		
lambda NM1149	vector for *R. fredii* USDA191 gene library	16
lambda NM1149.21A	lambda NM1149 with *exo* from *R. fredii* USDA191	this study
lambda NM1149.22E	lambda NM1149 with *exo* from *R. fredii* USDA191	„

Abbreviations; Kn, kanamycin; St, streptomycin; Na, nalidixic acid; Nm, neomycin; Cm, chloramphenicol; Ap, ampicillin; Tc, tetracycline.

Gel permeation chromatography: Bio-Gel A-5m (BioRad) column (1.6x40 cm) were loaded with 1 ml of EPS samples and eluted with elution buffer at 4°C. Elution rate was approximately 15 min/ml and fraction volume was 1 ml. Hexose content in each fraction was determined by the anthrone method (29).

Triparental mating: Recombinant plasmids carrying *R. meliloti exo* genes were transferred individually to *R. fredii exo* mutants by triparental mating with helper plasmid pRK2013 using a filter mating technique (9). Transconjugants were screened on MSY agar (24) containing nalidixic acid, kanamycin sulfate, and tetracycline hydrochloride.

Preparation of chromosomal DNA: *Rhizobium* strains were grown at 30°C in 50 ml of MG broth (31) until stationary growth phase. Cells were then pelleted by centrifugation, resuspended in 5 ml of 10mM tris[hydroxymethyl] aminomethane-1mM ethylenediaminetetraacetic acid (TE) buffer (26), and lysed by the addition of SDS and proteinase K to final concentrations of 0.5% and 100 μg/ml, respectively. Sodium chloride was added to make a final concentration of 0.7 M. All of the cellular debris was precipitated by CTAB leaving only the nucleic acids in solution. This mixture was then extracted with phenol and chloroform (26). The genomic DNA in the aqueous phase was recovered by ethanol precipitation, dried, and redissolved in TE.

Preparation of plasmid DNA: For small scale preparation of plasmid DNA, the alkaline-SDS procedure of Birnboim (6) was used. In addition, CsCl-EtBr gradient ultracentrifugation was employed for large scale purification of plasmid DNA (26).

Preparation of lambda phage DNA: *E. coli* SM32 and NM514 were used as host cells, respectively, for lambda NM1149 and lambda NM1149 clones. Lambda phage was purified by CsCl gradient ultracentrifugation and the DNA was then extracted according to Maniatis et al. (26).

Preparative agarose gel electrophoresis: Low melting temperature agarose gel electrophoresis was performed to isolate desired DNA fragments according to Maniatis et al. (26). Whenever necessary, DNA was further purified with elutip-d column according to manufacturer's procedure (Schleicher & Schuell).

Lambda gene library construction: *R. fredii* USDA191 chromosomal DNA was digested by *Eco*RI, ligated into *Eco*RI digested lambda NM1149 DNA using T4 DNA ligase, and then the DNA was packaged *in vitro* into phage particles using a lambda DNA packaging system (Promega). Recombinant phage constituted 0.5% of total phage population based upon phage titers between *E. coli* SM32 and NM514. Wild-type lambda phage gave 0.01% of background titer on NM514 compared with that on SM32. This gene library was then screened for wild-type *R. fredii exo* genes without further amplification.

Transformation: *E. coli* HB101 or DH5α were used as recipient cells. They were made competent by $MgCl_2$ and $CaCl_2$ treatments as follows. Cells were grown in LB until OD_{600}=0.4, pelleted by centrifugation, washed with ice-cooled 0.1 M $MgCl_2$, and then resuspended in 0.1 M $CaCl_2$ on ice for 30 min. Cells were again pelleted by centrifugation, resuspended in 1/10 volume with 0.1 M $CaCl_2$, and kept at 4°C for 1 hr. These competent cells were mixed with DNA (0.1-1.0μg), heated at 37°C for 1 min, incubated for 30 min. on ice, and then heated at 42°C for 5 min. After addition of 10 volumes of LB, the transfomation mixtures were incubated at

37°C for 1 hr. Aliquots of the cell suspension were spreaded on appropriate control and selection plates.

DNA-DNA hybridization: DNA was digested with restriction enzymes, separated on the agarose gel, and transfered to nitrocellulose filter by the method of Southern (26). For plaque hybridization, phage was grown on *E. coli* NM514 bacterial lawn, and phage DNA was transfered to a nitrocellulose filter by usual procedures (26). Probe DNA was labeled with biotin-11-dUTP or biotin-7-dATP by nick translation (BRL). Hybridization conditions and detection protocols for biotinylated probe DNA were as described in manufacturer's recommendations (BRL).

Results

Tn5 insertion of the *exo* mutants of *R. fredii.*

We previously concluded (Chapter 1) that the five *exo* mutants contained Tn5 inserts within their chromosome. The source of Tn5 was the plasmid pRK602 (Fig. 1A). In order to determine whether each Tn5 insertion event was unique, chromosomal DNAs of these *exo* mutants were digested with a restriction enzyme, *Eco*RI, and probed with a Tn5 probe.

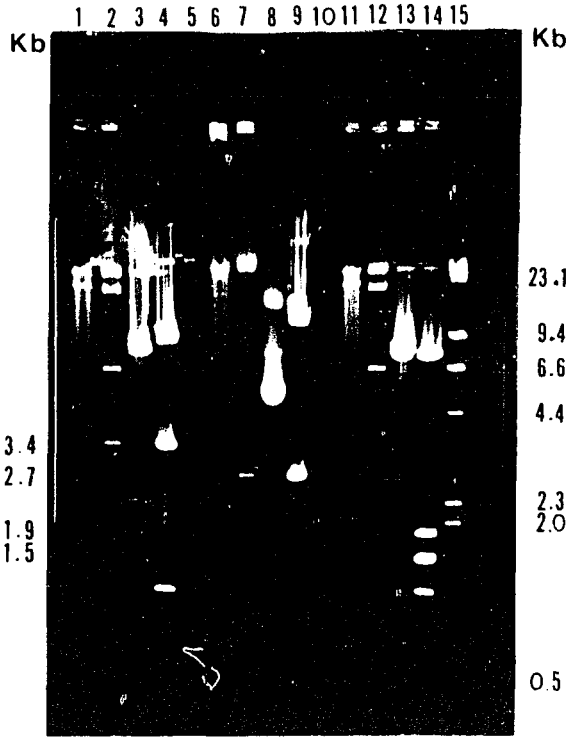
Total DNA from each mutant was digested completely with *Eco*RI which does not cut Tn5 (Fig.1B). DNA fragments were separated on a 0.6% agarose gel, transferred to a nitrocellulose filter, and then hybridized with a Tn5 containing DNA probe, pSUP2021 (Fig.1A) or a control DNA probe, pSUP202 (Fig.1A) without Tn5. The probe pSUP2021 hybridized to DNA fragments from each of the five *exo* mutants (Fig.2A/a-E/e) but not to any DNA fragments of the chromosomal digests of wild-type *R. fredii* USDA191 (Fig.2G/g) or nalidix acid resistant YKL999 (Fig.2F/f). Control probe, pSUP202 DNA, did not hybridize to any mutant's chromosomal DNA fragment (Fig.3A/a-E/e), although it did hybridize with pSUP2021 (Fig.3H/h) as expected. The sizes of *Eco*RI fragments containing Tn5 were larger than 5.7Kb (Tn5 size) but smaller than 14 Kb. Only YKL285 (*exo3*) (Fig.2C/c) and YKL293 (*exo5*) (Fig.2A/a) appeared to have inserts within the same DNA region because both had a 13.5 Kb *Eco*RI fragment which hybridized to the Tn5 probe DNA.

Comparison of extracellular polysaccharides of *R. fredii* and *R. meliloti.*

In order to compare the extracellular polysaccharides, cells of *R. fredii* YKL999 and *R. meliloti* 1021 were grown under same conditions in MG broth (33). Extracellular polysaccharides from the culture supernatants were then fractionated on a Biogel A-5m column.

Fig. 1. Transposon Tn5 and Tn5-containing plasmids. (A) Restriction enzyme digestion patterns of plasmids that were used for transposon mutagenesis (pRK602), DNA hybridization (pSUP2021 and 202), and conjugation (pRK2013). Note, Tn5 internal DNA fragments are indicated on the left side. DNAs of plasmids were digested with *Hin*dIII (lanes 1-4), *Bgl*II (lanes 6-9), or double digested with *Bam*HI and *Hin*dIII (lanes 11-14). lanes 1, 6, and 11, pRK2013; lanes 2, 7, and 12, pRK602; lanes 3, 8, and 13, pSUP202; lanes 4, 9, and 14, pSUP2021; lane 15, *Hin*dIII digested lambda DNA standards with DNA sizes indicated at right side. (B) A restriction map of transposon Tn5 (32).

A


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Kb
Kb
23.1
9.4
6.6
4.4
3.4
2.7
2.3
2.0
1.9
1.5
0.5

B

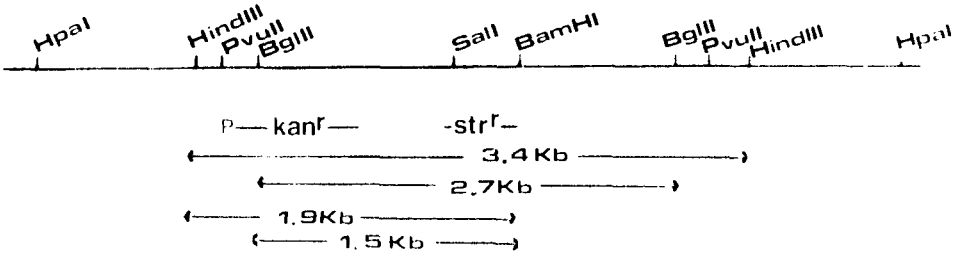

HpaI
HindIII
PvuII
BglII
SalI
BamHI
BglII
PvuII
HindIII
HpaI
P— kanr —
-strr —
3.4 Kb
2.7Kb
1.9Kb
1.5 Kb

Fig. 2. Southern blot analysis of *R. fredii* total DNA with labeled pSUP2021. DNA from each strain was digested with *Eco*RI, separated by agarose gel electrophoresis, transferred to nitrocellulose filter, and then hybridized with biotin-labeled pSUP2021. Lanes A-H, agarose gel; lanes a-h, corresponding hybridization; lane A/a, YKL293(*exo*5); lane B/b, YKL288(*exo*4); lane C/c, YKL285(*exo*3); lane D/d, YKL257(*exo*2); lane E/e, YKL224(*exo*1); lane F/f, YKL999(wt); lane G/g, USDA191; lane H/h, lambda DNA digested with *Hin*dIII.

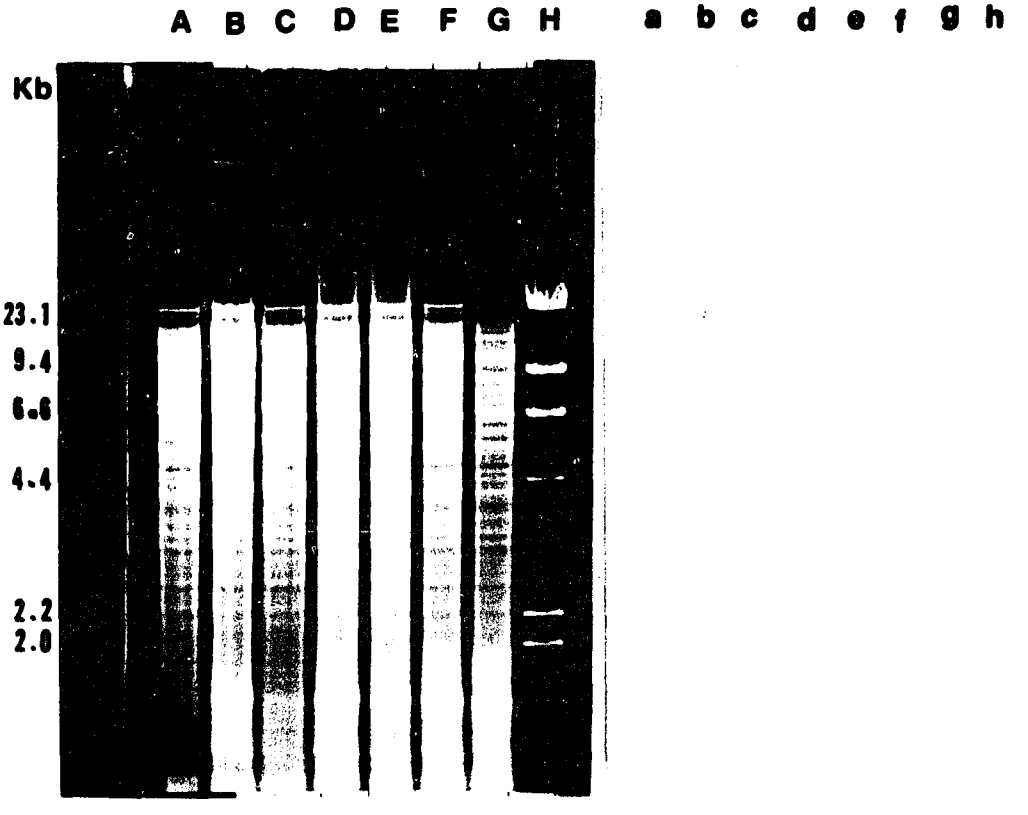
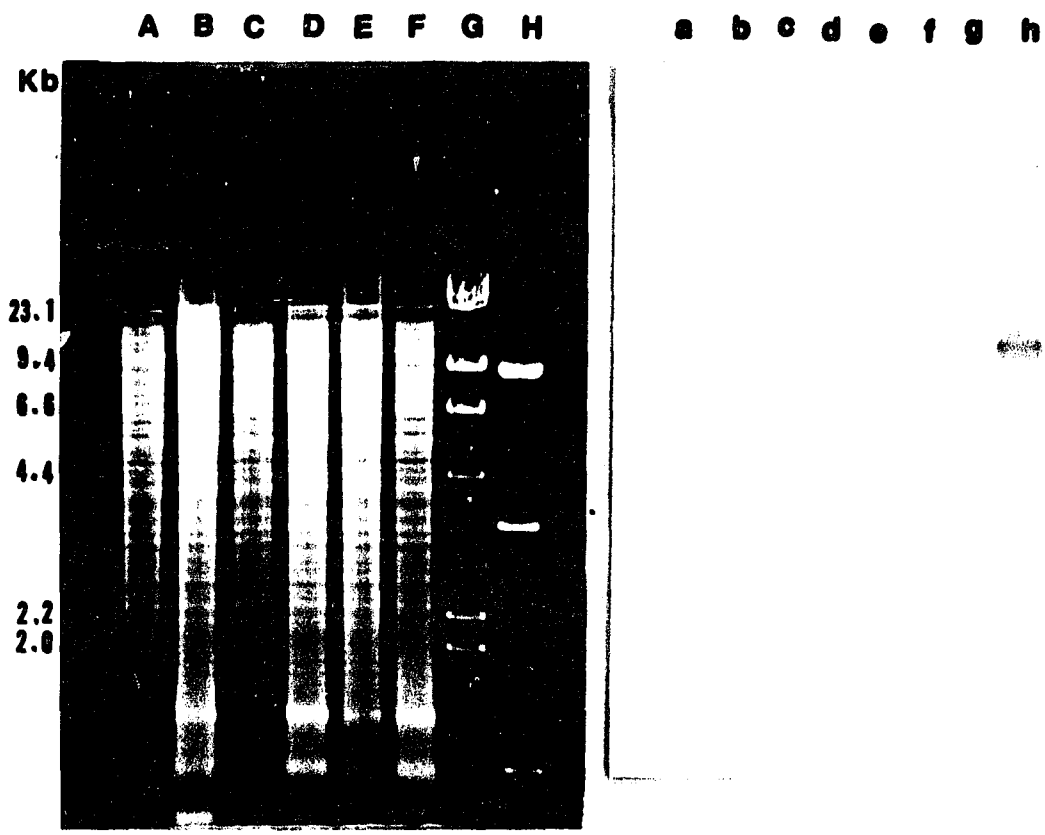

A B C D E F G H a b c d e f g h
Kb
23.1
9.4
6.6
4.4
2.2
2.0

Fig. 3. Southern blot analysis of *R. fredii* total DNA with labeled pSUP202. Total DNA from each strain was digested with *Eco*RI, separated by agarose gel electrophoresis, transferred to nitrocellulose filter, and then hybridized with biotin-labeled pSUP202. Lanes A-H, agarose gel; lanes a-h, corresponding hybridization; lane A/a, YKL293(*exo*5); lane B/b, YKL288(*exo*4); lane C/c, YKL285(*exo*3); lane D/d, YKL257(*exo*2); lane E/e, YKL224(*exo*1); lane F/f, YKL999(wt); lane G/g, lambda DNA cut with *Hin*dIII; lane H/h, pSUP2021 cut with *Hin*dIII


A B C D E F G H
a b c d e f g h
Kb
23.1
9.4
6.6
4.4
2.2
2.0

R. meliloti (Fig.4B) as has been previously demonstrated (21) produced polysaccharide that fractionated into two major peaks on a Biogel A-5m column. The larger molecular weight fractions (leading peak) were composed of succinoglycans and the smaller molecular weight fractions (tailing peak) primarily composed of B-glucans (21). *R. fredii* YKL999 (Fig.4A) also produced a neutral glucan peak with fractions corresponding to elution volumes seen with *R. meliloti* extracellular polysaccharide. Also, *R. fredii* had a large peak of acidic, large molecular weight polysaccharides. In fact, *R. fredii* compared with identical culture supernatants of *R. meliloti* had four times more anthrone reactible extracellular polysaccharide. Moreover, *R. fredii* was more mucoid and translucent on MSY (24) agar plates than *R. melioti* 1021.

Complementation of *R. fredii exo* mutants with plasmids containing *R. meliloti exo* genes.

Derivatives of the broad host range plasmid pLAFRI, which contained *R. meliloti exo* genes (Table 1;22) were introduced individually into our *exo* mutants of *R. fredii* using triparental matings (9). The correction of exopolysaccharide-deficiency was examined on MSY (24) agar plates.

YKL288 (*exo*4) and YKL293 (*exo*5) were restored in exopolysaccharide production, thus indicating complementation by plasmids pD56 (*exo*F/B) (Fig.6G) and pD15 (*exo*C) (Fig.5C), respectively. Transconjugants, YKL288/pD56 and YKL293/pD15, produced 0.66 and 0.86 of the acetone precipitable extracellular polysaccharides compared with wild type YKL999 in MG broth (33).

Table 2 summarizes the complementation experiment. Interestingly, YKL285 (*exo*3), YKL257 (*exo*2), and YLK224 (*exo*1) were not affected by the introduction of any *exo* plasmids from *R. meliloti*.

Fig. 4. Elution profiles of extracellular polysaccharides on Biogel A-5m column from *R. fredii* YKL999 (A) and *R. meliloti* 1021 (B). Vo denotes void volume of the column (1.6x40 cm).

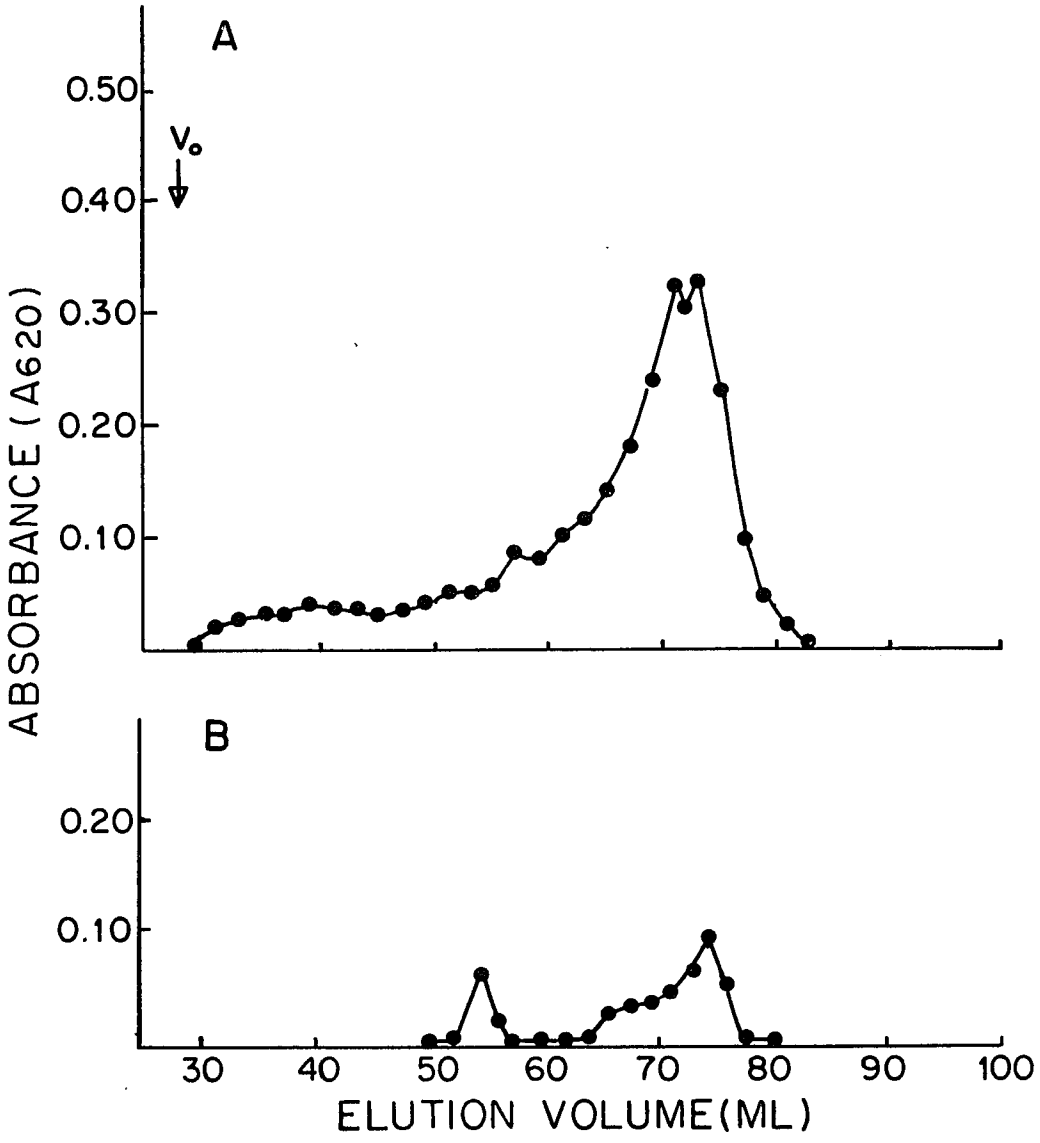

A
B
0.50
0.40
0.30
0.20
0.10
V_o
ABSORBANCE (A620)
30
40
50
60
70
80
90
100
ELUTION VOLUME(ML)

Fig. 5. The phenotypes of *R. fredii* YKL293 upon the introduction of *R. meliloti exo* gene clones. Cells were grown on MSY plate containing nalidixic acid. A, YKL293(*exo*5); B, YKL999(wt); C, YKL293/pD15; D, YKL293/pD34; E, YKL293/pD5; F, YKL293/pD2; G, YKJL293/pD56.


A
B
C
D
E
F
G
H

Fig. 6. The phenotypes of *R. fredii* YKL288 upon the introduction of *R. meliloti exo* gene clones. Cells were grown on MSY plate containing nalidixic acid. A, YKL288(*exo*4); B, YKL999(wt); C, YKL288/pD15; D, YKL288/pD34; E, YKL288/pD5; F, YKL288/pD2; G, YKL288/pD56.

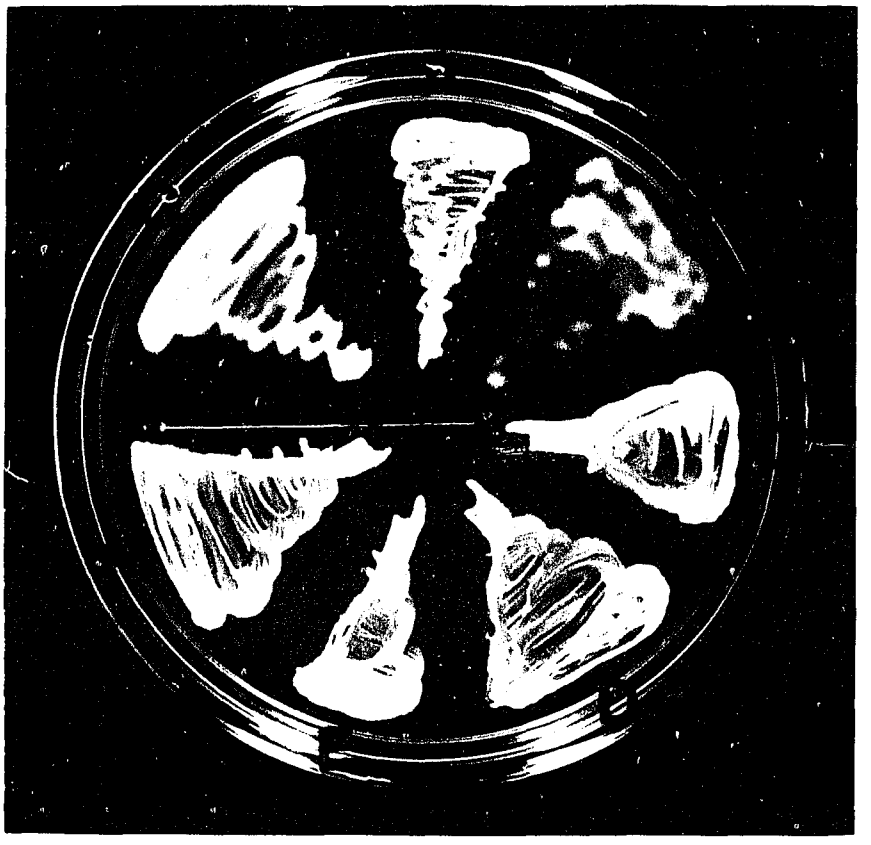


Table 2. Complementation of *R. fredii exo* mutants by *exo* cosmid clones of *R. meliloti*

	exo transfered clones				
strains	pD56 (*exo*F/B)	pD2 (*exo*B)	pD5 (*exo*D)	pD34 (*exo*A)	pD15 (*exo*C)
YKL293(*exo*5)	-	-	-	-	+[a]
YKL288(*exo*4)	+[b]	-	-	-	-
YKL285(*exo*3)	-	-	-	-	-
YKL257(*exo*2)	-	-	-	-	-
YKL224(*exo*1)	-	-	-	-	-

a, 86%; b, 66% production of the wild-type YKL999 EPS when cells were grown in MG broth. EPS was precipitated from the culture supernatant by addition of two volumes of acetone, and quantified by anthrone method (29).

Cloning *exo* gene of *R. fredii* YKL293.

The exopolysaccharide mutant, YKL293 (*exo*5), was the most interesting *exo* mutant because it exhibited a somewhat pleiotropic phenotype. It grew slowly compared to other mutants and the wild type; it was defective in the synthesis of both extracellular polysaccharide fractions, the acidic polysaccharides and neutral glucans (see Chapter 1). In addition, we demonstrated above that its exopolysaccharide defect can be complemented by an *exo*C clone from *R. meliloti*.

First, a chromosomal *exo*5 gene fragment interrupted by the Tn5 insertion within this mutant was cloned. Chromosomal DNA was digested with *Bam*HI to generate DNA fragments with Tn5 encoded kanamycin resistance gene at one end. This chromosomal DNA digest was ligated with pUC19 DNA, predigested with the same restriction enzyme, using T4 DNA ligase. The ligated DNA was used for transformation of *E. coli* DH5a . White colonies on X-gal plates containing kanamycin and carbenicillin were isolated and purified. Plasmid pLYK5293 DNA was prepared from these transformants. Recombinant plasmid pLYK5293 contained an internal DNA fragment of Tn5 coding for kanamycin resistance. pLYK5293 DNA was digested with several restriction enzymes (Fig.7A) to determine the orientation and the extent of the cloned DNA. The sizes of various restriction DNA fragments of pLYK5293 are presented in Table 3. A restriction map of plasmid pLYK5293 derived from Table 3 data is presented in Fig. 7B. A 3 Kb chromosomal DNA (*exo*5 DNA sequences) that flanks one side of the Tn5 gene was cloned.

To isolate the wild type *exo*5 gene coding sequence, biotin-labeled DNA of pLYK5293 was used as a probe to screen a *R. fredii* USDA191 gene library constructed with lambda vector NM1149 . On first screening by "plaque hybridization" (26), two positive clones (21A and 22E) were chosen out of 10,000 recombinant plaques (Fig.8A). On second screening, the above clones (21A and

Fig. 7. Restriction map of plasmid pLYK5293. (A) Restriction enzyme digestion patterns of pLYK5293. The plasmid DNA was digested with several restriction enzymes and separated on a 0.6% agarose gel. lane 1, lambda DNA standard digested with *Eco*RI; lane2, pLYK5293 uncut; lane 3, *Sal*I ; lane 4, *Xho*I and *Eco*RI; lane 5, *Xho*I; lane 6, *Bgl*II and *Xho*I; lane 7, *Bgl*II; lane 8, *Bam*HI and *Bgl*II; lane 9, *Hin*dIII; lane 10, *Bam*HI and *Hin*dIII; lane 11, *Eco*RI; lane 12, *Bam*HI and *Eco*RI; lane 13, *Bam*HI; lane 14, pLYK5293 uncut; lane 15, lambda DNA standard digested with *Hin*dIII. (B) Restriction map of pLYK5293. This map is derived from above digestion patterns which are summarized in Table 3. All the restriction sites except by *Sal*I within *R. fredii* chromosome fragment are indicated. B, *Bam*HI; G, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; X, *Xho*I; Tn5', a part of Tn5 fragment containing kanamycin resistance gene; Rfexo, *R. fredii* DNA sequences flanking Tn5 from YKL293(*exo5*).

A


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Kb
Kb
21.2
7.4
5.7
4.9
3.5
23.1
9.4
6.6
4.4
2.3
2.0

B

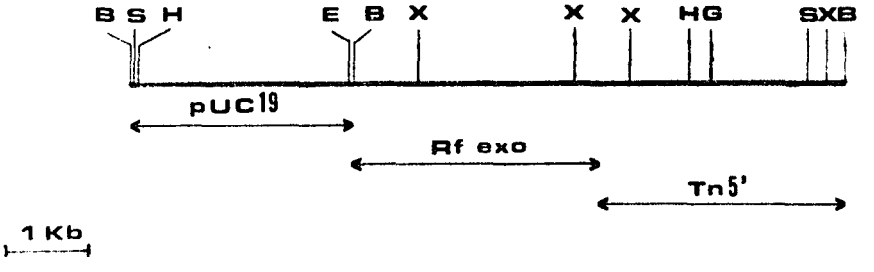

B S H
E B X
X
X
HG
SXB
pUC19
Rf exo
Tn5'
1 Kb

Table 3. Restriction fragments of plasmid pLYK5293

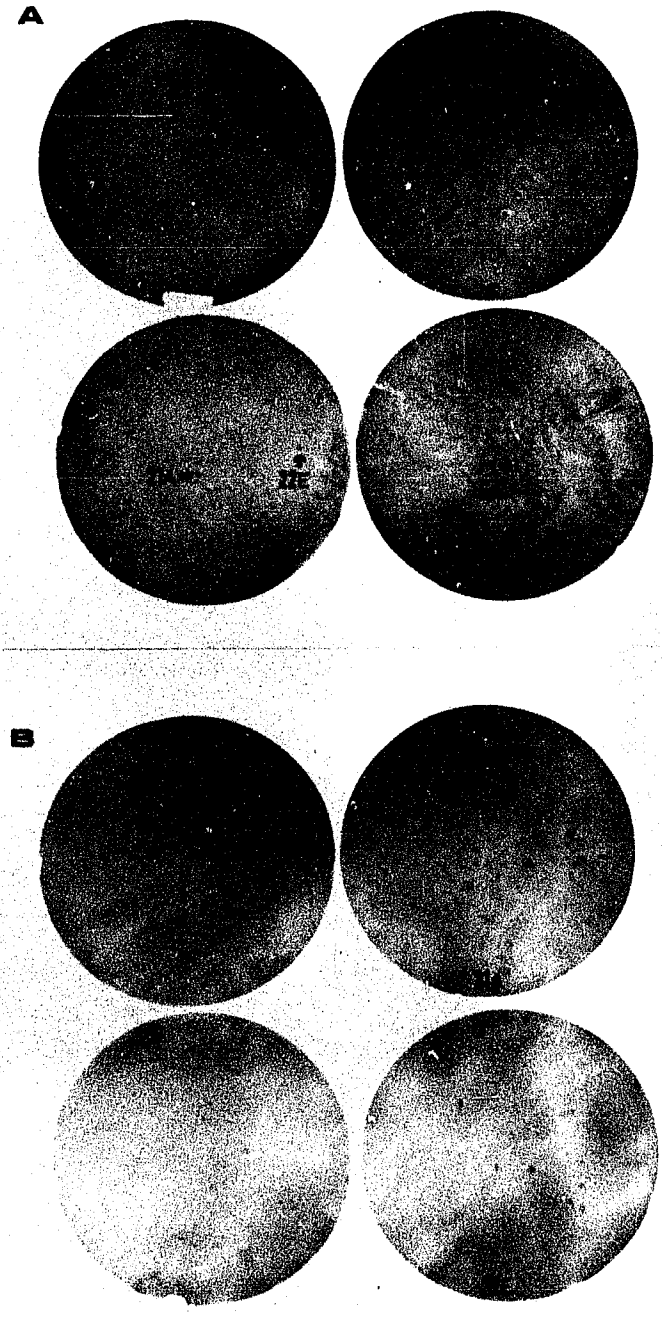
	Restriction enzymes										
	Sal	Xho Eco	Xho	Bgl Xho	Bgl	Bam Bgl	Hin	Bam Hin	Eco	Bam Eco	Bam
					8.7				8.7		
							6.7				
										6.0	6.0
						4.5					
								4.0			
DNA			3.5	3.5							
fragments	3.0										
(Kb)**		2.7				2.7		2.7		2.7	2.7
	2.6										
		2.4	2.4								
		2.0	2.0	2.0							
							1.9	1.9			
						1.5					
	1.35		1.3								
				1.0							
		0.8									
		0.7	0.7	0.7							
	0.6										
	0.4*										
								0.03*		0.02*	

Abbreviations; Sal, *Sal*I; Xho, *Xho*I; Eco, *Eco*RI; Bgl, *Bgl*II; Hin, *Hin*dIII; Bam, *Bam*HI.

* The DNA fragments which should have migrated off the gel were not detected in Fig. 7A. The sizes of these three DNA fragments were calculated from the published restriction maps of plasmid pUC19 and transposon Tn5.

** The size of plasmid pLYK5293 was 8.7 Kb. The sizes of all DNA fragments, except those three mentioned above,
were estimated from the migration distances on a 0.6% agarose gel (Fig.7A).

Fig. 8. Plaque DNA hybridization of filters of a *R. fredii* USDA191 gene library screened with biotin-labeled pLYK5293. Phage were plated on *E. coli* NM514 bacterial lawns, transferred to nitrocellulose filters, and hybridized with biotin-labeled plasmid pLYK5293. (A) First round selection of plaques, 21A and 22E. (B) Second round passage and plaque purification.


A
B

22E) were purified by sequential dilution and plating on a NM514 lawn (Fig.8B). Phage DNA was extracted and purified from these two clones, lambda NM1149. 21A and 22E. The DNA was digested with *Eco*RI and DNA fragments were separated by agarose gel electrophoresis, transferred to a nitrocellulose filter, and then hybridized with a biotin-labeled pLYK5293 DNA probe. The probe hybridized to only two cloned DNA inserts; the 2.5 Kb *Eco*RI DNA fragments of NM1149.21A (Fig.9E/e-H/h) and the 3.3 Kb *Eco*RI DNA fragments of NM1149.22E (Fig.9A/a-D/d).

Hybridization of *exo* DNA sequences.

Plasmid pLYK5293 contained DNA sequences from a mutant YKL293 (*exo*5) (Fig.7B). Since this mutant was complemented by a *R. meliloti exo*C plasmid clone, we decided to see whether homology exists between the two genes and the other *R. meliloti exo* genes. Plasmid DNAs of pLAFRI clones containing the *exo* genes were digested with *Eco*RI. DNA fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose filters. Homology was tested by DNA-DNA hybridization with biotin-labeled pLYK5293 DNA as a probe. Even though plasmid pD15 (*exo*C) (Fig.A/a) was expected to be homologous to the probe pLYK5293, plasmids pD56 (*exo*F/B) (Fig.10E/e), pD2 (*exo*B) (Fig.10D/d), and pD5 (*exo*D) (Fig.10C/c) showed positive hybridization (Fig.10C/c), while the rest of plasmids did not have any detectible hybridization. As a positive control for the probe, a chromosomal DNA digest of YKL293 had a 13.5 Kb *Eco*RI fragment hybridized to plasmid pLYK5293 (Fig.10F/f). In addition, the wild-type strain USDA191 had a positively hybridizing 7.8 Kb *Eco*RI fragment (Fig.10H/h) which corresponded to the expected size, 13.5 Kb minus 5.7 Kb (Tn5 size).

Fig. 9. Southern blot analysis of lambda NM1149. 21A and 22E DNAs with biotin-labeled pLYK5293. *Eco*RI digested DNAs were separated by a 0.6% agarose gel electrophoresis, blotted onto a nitrocellulose filter, and hybridized with biotin-labeled plasmid pLYK5293. lanes A-I, agarose gel; lanes a-i, corresponding hybridization; lanes A/a-D/d, lambda NM1149. 22E; lanes E/e-H/h, lambda NM1149. 21A; lane I/i, *Eco*RI digested lambda DNA standard.

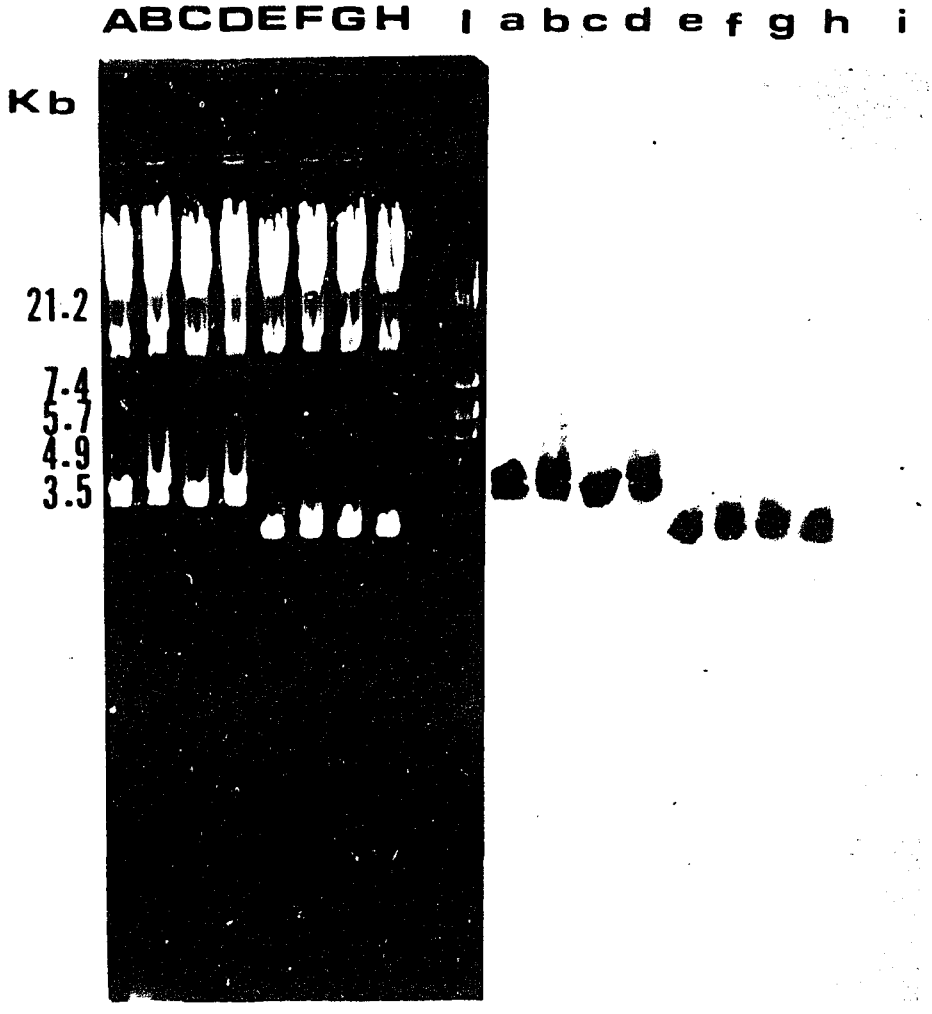
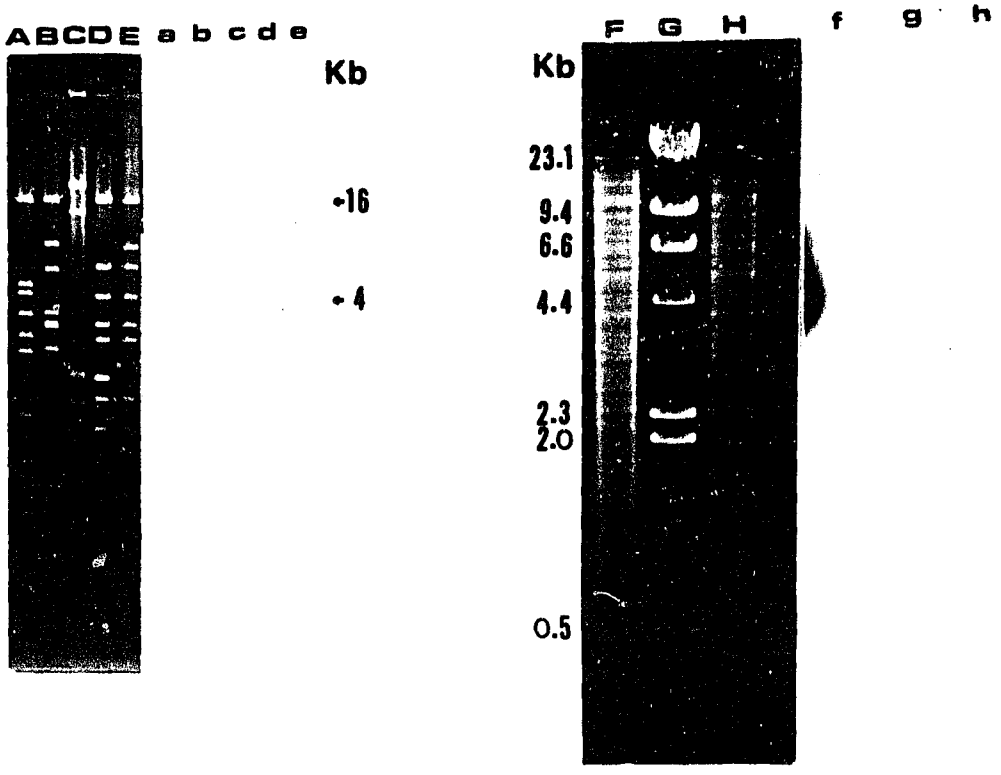

A B C D E F G H I a b c d e f g h i
Kb
21.2
7.4
5.7
4.9
3.5

Fig. 10. Southern blot analysis of *R. meliloti exo* gene clones and *R. fredii* genomic DNA with biotin-labeled pLYK5293. DNAs were digested with *Eco*RI, separated by 0.6% agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized with biotin-labeled plasmid pLYK5293. lanes A-H, agarose gel; lanes a-h, corresponding hybridization; lane A/a, pD15; lane B/b, pD34; lane C/c, pD5; lane D/d, pD2; lane E/e, pD56; lane F/f, YKL293(*exo5*); lane G/g, lambda DNA digested with *Eco*RI; lane H/h, USDA191.


A B C D E a b c d e
Kb
16
4
F G H f g h
Kb
23.1
9.4
6.6
4.4
2.3
2.0
0.5

Discussion

The major question asked in this paper is whether *exo* mutants of *R. fredii* are related to the *exo* mutants of *R. meliloti*. There were several reasons we expected that this might be the case, even though they form symbiotic relationships with different legumes. When plasmids containing *R. meliloti exo* genes were introduced into our five *exo* mutants, exopolysaccharide deficiency of YKL288 (*exo*4) was restored by plasmid pD56 (*exo*F/B), but not by plasmid pD2 (*exo*B) (Fig.6) and plasmid pD15 (*exo*C) also restored exopolysaccharide production in YKL293 (*exo*5) (Fig.5). Three *R. fredii exo* mutants were not complemented by any of the *R. meliloti exo* gene containing plasmids. These three mutants may contain mutations of exopolysaccharide synthesis that are unique for *R. fredii*.

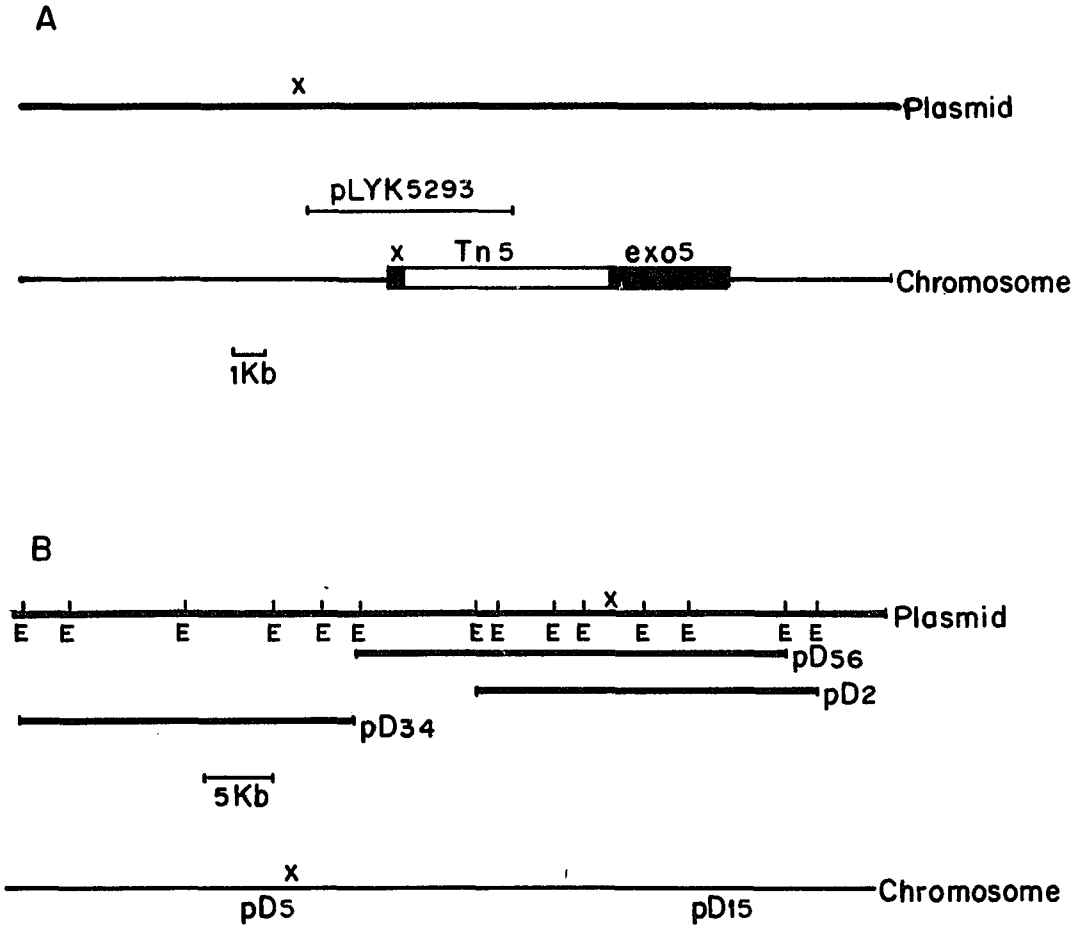
The phenotypes of the two *exo* mutants of *R. fredii* which were complemented are considerably different. YKL288 is defective in synthesis of the large molecular weight (M.W.) acidic polysaccharides but still synthesized about 25% of normal neutral glucans. A similar phenotype was observed with an *exo*F mutant of *R. meliloti* 1021 which was defective in succinoglycan production but still synthesized β-glucan (21). YKL293 has a pleiotropic mutation that affects strain growth rate and results in a severe loss of both the large M.W. acidic exopolysaccharides and low M.W. neutral glucans. Interestingly, the *exo*C mutant of *R. meliloti* is also pleiotropic ,in that it grows slower than normal and also produces little if any acidic or neutral polysaccharides (21). Furthermore, recent studies of *Agrobacterium tumefaciens* indicate that it has a gene, *psc*A, that is closely related to *exo*C of *R. meliloti* and the mutant also is defective in β-glucan production (19). It is likely that both *Rhizobium* species have common pathways in exopolysaccharide synthesis.

Both *exo*D and *exo*C gene loci of *R. meliloti* were located on the chromosome, whereas other *exo* loci, *exo*F, *exo*B, and *exo*A were on a symbiotic

plasmid in *R. meliloti* (13). The linkage map of *exo*C and *exo*D has not been reported. We attempted to clone the *R. fredii exo*5 gene interrupted by Tn5 in YKL293, which could be complemented with the plasmid containing *exo*C gene of *R. meliloti.* Recombinant plasmid pLYK5293 (Fig.7B), obtained from kanamycin and carbenicillin resistant transformants, had chromosomal DNA sequences flanking Tn5 on one side. We expected homology with *R. meliloti exo*C gene. However, Southern filter hybridization of *R. meliloti exo* clones with pLYK5293 DNA probe revealed cross hybridization with the *exo*F, *exo*B, and *exo*D clones. No hybridization signal with *R. meliloti exo*C clone was detected (Fig.10). This cross hybridization with *exo*D but complementation with *exo*C implies a possible close location of *exo*D and *exo*C gene homologues in *R. fredii* USDA191. And the positive hybridization of the probe with *exo*F/B and *exo*D genes suggests repeated DNA sequences in *R. meliloti*; one in the second symbiotic plasmid and the other in the chromosome (Fig.11B). The absence of detectible hybridization between probe pLYK5293 and *exo*C clones might result from the possiblity that the probe DNA contains too small a DNA fragment to show hybridization with *exo*C DNA due to the insertion of Tn5 into the terminal region of *the exo*C homologue (*exo*5) in YKL293 (Fig.11A). Alternatively, Tn5 may be inserted into a regulatory DNA sequences which may not be contained in the particular *exo*C gene clone of *R. meliloti.*

To pick up the wild type *exo*5 gene, a *R. fredii* USDA191 gene library in lambda NM1149 was screened by plaque hybridization with a labeled pLYK5293 DNA probe. Two positively hybridizing plaques (lambda NM1149. 21A and 22E) were obtained. However, the sizes of the DNA inserts in above clones (Fig.9), 2.5 and 3.3 Kb, were not the expected 7.8 Kb *Eco*RI DNA fragment disrupted by Tn5 insertion in YKL293 (Fig.10H/h). They also differed from each other. These two *Eco*RI DNA fragments were not detectible by DNA hybridization in a *Eco*RI genomic

Fig. 11. Speculation about *exo*5 locus and duplicated DNA sequences. (A) Transposon Tn5 is speculated to be inserted into the terminal region of *exo*5 gene in *R. fredii* YKL293. (B) The linkage map of *R. meliloti exo* genes are from Long, et al. (25). On a symbiotic plasmid are located *exo*F/B (pD56), *exo*B (pD2), and *exo*A (pD34) genes. The linkage map of *exo*C (pD15) and *exo*D (pD5), which has not been reported, were mapped on the chromosome. Restriction sites by *Eco*RI are indicated as letter E. The X marks in (A) and (B) indicate the approximate locations of hybridizable DNA sequences contained on plasmid pLYK5293. A X mark on the plasmid of *R. fredii* (A) indicates a possible duplicated region of homology.


A
x
Plasmid
pLYK5293
x
Tn5
exo5
Chromosome
1Kb
B
x
E E E E E E E E E E E E E
Plasmid
pD56
pD2
pD34
5Kb
x
Chromosome
pD5
pD15

DNA digest with pLYK5293 probe (Fig.10H/h). The two DNA fragments in NM1149. 21A and 22E most likely came from other DNA site(s) rather than the *exo*5 locus interrupted by Tn5 in YKL293. This finding suggests that the *exo*5 gene (or its regulatory sequence) is duplicated in *R. fredii*. Many genes including regulatory elements are repeated in several *Rhizobium* species (27). Symbiotic regulatory genes such as *nif* and *nod* upstream promoter regions are duplicated (5,31). In addition, duplicated structural genes for *nif* (4) and *nod* (2) have been found in *R. fredii*.

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CONCLUSIONS

Exopolysaccharide-deficient (*exo*) mutants of *R. fredii* USDA191 were obtained by Tn5 insertion mutagenesis to study the functional roles of exopolysaccharide in the nodulation of soybeans. Five mutants, out of eighty-five *exo* mutants isolated, were chosen based upon their unique phenotypes on agar plates and these were further examined for exopolysaccharide production and soybean nodulation abilities.

Exopolysaccharides were analyzed by BioGel A-5m and DEAE-Sephadex A-50 column chromatography and thin-layer chromatography. Nodulation abilities were tested by innoculating soybean species, *G. soja* and *G. max* cv. Peking, with each mutant and wild-type strain of *R. fredii*. Nodulation of *G. soja* by YKL999 (wt) was less efficient than nodulation of *G. max*. The wild-type induced a large number of atypical nodules on *G. soja* and few typical nodules; whereas, it induced only typical nodules on *G. max* cv. Peking. Thus, *R. fredii* USDA191 had a preference for Peking rather than for *G. soja*.

*Exo*1 mutant (YKL224) produced 235% of neutral glucans compared with the wild type YKL999 but was deficient in the synthesis of the large M.W. acidic exopolysaccharides. *Exo*2 mutant (YKL257) was only partially nonmucoid in appearance on agar plate and still synthesized about half of the normal level of exopolysaccharides. Nodulation of Peking by *exo*1 and *exo*2 mutants were extremely inefficient. Possibly *exo*1 mutant may have a cell membrane change because it has a high level of neutral glucan (normally found in periplasmic space) that is excreted. Also *exo*2 may be defective in the modification of polysaccharides. The other two *exo* mutants, *exo*3 (YKL285) and *exo*5 (YKL293), were still able to induce nodule formation even though they lost the ability to synthesize exopolysaccharides completely. This finding suggests that exopolysaccharides are not absolutely

required for nodulation by *R. fredii* USDA191. The root nodule formation by *exo*4 (YKL288), that had only 25% of the normal level of neutral glucans, also supported a less stringent role for exopolysaccharides in nodulation. However, these three *exo* mutants (*exo*3, *exo*4, and *exo*5) did not induce as many nodules as the wild-type, YKL999. Thus, deficiency in exopolysaccharide production does reduce nodulation ability. It is likely that exopolysaccharide functions may differ between *R. fredii* USDA191 and *R. meliloti* 1021. In *R. meliloti*, one of the best characterized *Rhizobium* species, exopolysaccharide production was found to be tightly linked with nodule invasion. Even a lack of proper modification of exopolysaccharides resulted in the formation of only atypical nodules. In order to better understand the functional roles of exopolysaccharide in nodulation process, it is necessary to see whether exogeneous addition of wild type exopolysaccharides to each innoculum of *exo* mutants abolish the defect of their nodulation abilities.

We tried to compare our *exo* mutants of *R. fredii* USDA191 with *exo* mutants of *R. meliloti* through gene complementation and DNA hybridization. Upon introduction of cloned *exo* genes of *R. meliloti* into our *exo* mutants, only *exo*4 and *exo*5 were complemented, respectively, by *exo*F and *exo*C gene clones in exopolysaccharide production. But the complementation of nodulation defects was not tested. It is expected that the *exo* genes of *R. meliloti* will restore the nodulation ability of those two mutants. This complementation data imply that two *Rhizobium* species share some common pathways in exopolysaccharide synthesis.

In order to compare DNA homology between two *Rhizobium* species, DNA sequences flanking Tn5 in *exo*5 mutant were cloned into a plasmid pUC19. The recombinant plasmid pLYK5293 had a 3Kb chromosomal DNA sequences in addition to a part of Tn5 coding kanamycin resistance. Southern hybridization analysis of *R. meliloti exo* gene clones with a plasmid pLYK5293 as a probe DNA revealed that *exo*F/B, *exo*B, and *exo*D clones did have DNA sequences which can

hybridize to the probe. *Exo*F and B genes are located on the second symbiotic plasmid and *exo*D gene on the chromosome. It appeared that the DNA sequences identified by pLYK5293 hybridization might be duplicated in *R. meliloti.* Interestingly, the DNA fragment that hybridized to the probe pLYK5293 was not part of structural gene for *exo*F or *exo*B. It may be another *exo* gene that has not been identified in *R. meliloti* and for which no mutation on this locus has been documented.

The same plasmid DNA, pLYK5293, was used as a probe to screen for the wild-type *exo*5 gene from a *R. fredii* USDA191 gene library using the lambda vector, NM1149. Positively hybridizing plaques, 21A and 22E, were isolated. Restriction analysis of the DNAs from these clones revealed that a 2.5 Kb and a 3.3 Kb *Eco*RI fragments were inserted within lambda NM1149. 21A and lambda NM1149. Surprisingly their base pair sizes did not match the expected wild type *exo*5 gene detected in *Eco*RI digests of chromosomal DNA. The size of wild-type *exo*5 gene was predicted to be 7.8 Kb. It is possible that *exo*5 gene or a fragment is also duplicated in USDA191. However it is premature to say because a supporting evidence is required. First of all, 2.5 Kb and 3.3 Kb DNA *Eco*RI fragments of lambda NM1149. 21A and 22E clones need to be tested for their hybridization with the 7.8 Kb *Eco*RI fragment of the wild-type *exo*5 gene. It is not known whether these two cloned inserts are linked together (part of same gene) or not. If they are linked, *Eco*RI enzyme may have cut within the *exo*5 gene. This can be tested by partial digestion of USDA191 genomic DNA with *Eco*RI and Southern blot hybridization using pLYK5293 as a probe DNA. It will be interesting to see whether putative wild type *exo* genes cloned in lambda NM1149. 21A and 22E originated from one of the indigenous plasmids of *R. fredii* or were chromosomal. Since large M.W. plasmids can be separated by in-well lysis technique on a agarose gel, DNA hybridization analysis with lambda NM1149. 21A and 22E probes can be

performed. Of course the definitive proof, the complementation of exopolysaccharide-deficiency in YKL293 by a cloned *exo5* gene need to be performed. This can be done by subcloning *exo5* gene in a broad host range plasmid such as pLAFRI and pRK290, and introducing them into YKL293 by conjugation or transformation.

VITA

Young Hwan Ko was born on May 19, 1956 (registered date, August 5, 1957) in Korea. He graduated from Cheju-Cheil High School in 1974. He received an B.S. degree in Food Science and Technology from Seoul National University, in 1978, and served in Marine Corps until 1980. In August, 1980, he entered Graduate School of Seoul National University, studied enzymology, and received an M.S. degree in Food Technology in 1982. During the years from 1982 until he came to U.S.A., he was employed as a researcher in the field of applied microbiology. In August, 1984, he entered the doctoral program in the Department of Microbiology at Louisiana State University. He is the father of Sooweon Sue, born May 17, 1983. He married Seonsil Choi in February, 1982. He is currently a candidate for the Doctor of Philosophy degree in Microbiology.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Young Hwan Ko

Major Field: Microbiology

Title of Dissertation: Isolation, characterization, and symbiotic effectiveness in soybeans of exopolysaccharide mutants of Rhizobium fredii USDA191.

Approved:

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F. Glen Hembry
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